Trichoderma & Gliocladium

Volume 1

Basic biology, taxonomy and genetics

Edited by

Christian P. Kubicek and **Gary E. Harman**



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Preface

Almost exactly 200 years ago, *Trichoderma* was introduced as a taxon to accommodate four fungal species, one of which actually was *Trichoderma viride,* while the others are now considered to be completely unrelated to this fungal genus. Over the next 150 years, *Trichoderma* was occasionally a subject of mycotaxonomists and a few others but did not attract the interest of other scientific disciplines. This situation changed dramatically during World War II when the US army was alarmed at the rate at which materials were rotting in tropical regions, particularly in the South Pacific. As a result, the army's Quartermaster Corps set up a long-range basic program to investigate the nature of rotting and thereby identified *Trichoderma "viride"* QM 6a as one of the most cellulolytic fungi from this region. It should be noted that, at this time, all *Trichoderma* strains were identified as *"T. viride"* and it took 20 more years before the unique biological nature of isolate QM 6a was recognized and named *"T. reesei"* in honour of its major investigator, Elwyn T.Reese. His pioneering work at the Natick Institute, in collaboration with Mary Mandels, made this fungus and several of its mutants a major subject of research on the biosynthesis, structure and mechanism of degradation of cellulose and other polysaccharides. It is due to their work that several other laboratories in the USA, Europe and Asia continued the exploitation of *Trichoderma's* cellulolytic system in the late 1960s, during the time of the first "oil shock".

At the same time, Rifai and Webster in Exeter (UK) attempted for the first time a taxonomic approach to *Trichoderma* and succeeded in defining nine species aggregates. The easy and inexpensive cultivation of most of these "species" has since attracted numerous other researchers interested in basic biological phenomena rather than cellulose degradation. One observation of major importance was the detection of the ability of selected species, most frequently published as *"T. harzianum"* or *"T. viride",* to antagonize the growth of plant pathogenic fungi and thus to act as biocontrol agents. Today this field has become the second basis on which most research on *Trichoderma* is built and has attracted numerous researchers worldwide.

When summarizing the last 20 years of fungal research, *Trichoderma* is probably one of the most successful newcomers; for example, in the area of enzyme production this fungus is second only to *Aspergillus*. At the time this book goes to press, there are more than 150 entries for *Trichoderma* in the gene data bank, and weekly records in the biological or agricultural section of Current Contents exceed those obtained for other well-known and intensively studied fungal genera such as *Penicillium* or *Neurospora*. Surprisingly, this strong interest in *Trichoderma* has to date not been reflected by the attempt to combine all the knowledge on *Trichoderma* in a monograph, whereas good monographs now exist for *Aspergillus, Penicillium* and several other plant pathogenic fungi. This lack of information is also reflected in the current existence of two separate and regularly held conferences, i.e., the TRICEL Conference (focusing on all aspects of *Tri*choderma *cel*lulases and other hydrolytic enzymes) and the *Trichoderma* and *Gliocladium* workshops (focusing mainly on taxonomy and various aspects of biological control).

The idea of the present monograph therefore was to present a comprehensive treatise of *Trichoderma* that covered all of the different aspects and can thus serve as a standard source to all working with any of these species. We have also chosen to include *Gliocladium* in the title and scope of the book since one of the key species— *Gliocladium virens*—is now considered to actually be *Trichoderma (T. virens)* and substantial information from biocontrol research has been obtained with this species. However, research in other areas with *Gliocladium* hardly exists and consequently most chapters of this book will deal with *Trichoderma* exclusively. We are grateful that Taylor & Francis have enthusiastically agreed to publish this work and we appreciate their help and collaboration on this project.

Lastly we would also like to thank all of our authors, who have been very co-operative in collaborating with us to produce this monograph.

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PART ONE

Basic biology

Morphology and identification of *Trichoderma*[1]

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1.1 Introduction

The genus *Trichoderma* is cosmopolitan in soils and on decaying wood and vegetable matter. Species of *Trichoderma* are frequently dominant components of the soil microflora in widely varying habitats. This may be attributable to the diverse metabolic capability of *Trichoderma* species and their aggressively competitive nature. Strains of *Trichoderma* are rarely associated with diseases of living plants, although an aggressive strain of *T. harzianum* causes a significant disease of the commercial mushroom (Volume 2, Chapter 12). Samuels (1996) provides a comprehensive review of the biology of *Trichoderma,* and of the technological exploitation of *Trichoderma* species for enzyme production and biological control.

Most strains of *Trichoderma* have not been associated with a sexual state and are believed to be mitotic and clonal. Species of *Hypocrea* and closely related genera in the Hypocreales have anamorphs referable to *Trichoderma,* and in recent years a growing number of teleomorphs in *Hypocrea* have been linked to commonly occurring *Trichoderma* anamorphs through macromolecular investigations. However, despite these significant advances in our knowledge of the genus, the taxonomy of *Trichoderma* is still rather incomplete, and the distinction of species in the genus *Trichoderma* remains problematic. A refined classification and identification is necessary for predictive indications about ecology (Danielson and Davey, 1973a,b; Davet, 1985; Claydon *et al.*, 1987; Papavizas, 1985; Widden and Hsu, 1987; Widden and Scattolin, 1988), toxicology (Almassi *et al.,* 1991; Brückner and Przybylski, 1984; Collins and Halim, 1972; Dennis and Webster, 1971; Dunlop *et al.*, 1989; Ghisalberti *et al.*, 1990; Ghisalberti and Rowland, 1993; Ghisalberti and Sivasithamparam, 1991; Hou *et al.,* 1972; Hutchinson, 1972; Okuda *et al.,* 1982; Shaw and Taylor, 1979; Simon *et al.,* 1988; Taylor, 1986; Webster and Lomas, 1964), and technology (Goldman and Lucio de Azevedo, 1987; Kubicek *et al.,* 1996; Morawetz *et al.,* 1992; Sternberg and Doval, 1980) as reviewed by Samuels (1996). A refined species concept may eventually be equivalent to the

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biological unit or "biological species", some of which can be correlated unequivocally with teleomorph species.

Because the equally relevant *Gliocladium virens* is now generally recognized as belonging to *Trichoderma,* this chapter is devoted solely to *Trichoderma*. The anamorph species *Gliocladium roseum,* commonly regarded as being associated with the teleomorph *Bionectria (Nectria) ochroleuca,* is unrelated to this genus. It is also unrelated to the type species of *Gliocladium* and its classification is not yet settled.

1.2 Taxonomic history

Although the genus *Trichoderma* has been known since the beginning of the 19th century and its association with teleomorphs in *Hypocrea* Fr. was recognized by the Tulasne brothers in 1865, its taxonomy has remained obscure until recent decades. Bisby (1939) thought that the morphological variation could be ascribed to a single species, *T. viride*. The first serious attempt to morphologically distinguish species, or rather "species aggregates", was made by Rifai (1969), who was aware that the nine taxa he distinguished were not biological entities correlated with single teleomorph species. These and a few additional species described subsequently were keyed out by Domsch *et al.* (1980). Teleomorph connections were established by means of ascospore isolates by Dingley (1957), and by Webster and coworkers (Rifai and Webster, 1966; Webster and Rifai, 1968). In Japan, a wealth of teleomorphs was studied thoroughly and described with cultural and anamorph characters by Doi (1969, 1972), but unfortunately no cultures have been preserved from this study. No further morphological differentiation of the anamorphs was attempted by Doi and Doi (1979, 1986). An intensified investigation of teleomorph taxonomy with cultural studies has been initiated by Samuels and coworkers (Samuels *et al.,* 1994; Samuels, 1996). Besides *Hypocrea, Trichoderma* anamorphs are known in the related genera *Podostroma* P. Karst. (Doi, 1967) and *Sarawakus* Boedijn (Rifai *et al.,* 1985). The most detailed morphological studies of the anamorphs were carried out by Bissett (1984, 1991a–c, 1992), who now distinguishes about 21 taxa in sect. *Pachybasium* and seven in sect. *Longibrachiatum,* while the remaining sections have not yet been treated in a comparable way. Such studies show that the delimitation of biological species is extremely difficult in this genus on morphological grounds alone.

Other taxonomic methods supplementary to morphology include studies of secondary metabolites, which show a great diversity in this genus (Okuda *et al.,* 1982). Physiological features detectable in microtiter plates may eventually provide a useful system for identification. Isoenzyme profiles have been used as an effective taxonomic tool (Leuchtmann *et al.,* 1996; Samuels *et al.,* 1994). Molecular data, particularly sequences of the ITS region of ribosomal DNA and fingerprinting techniques, have in recent years allowed the finest resolution of taxonomic entities (Fujimori and Okuda, 1994; Kuhls *et al.,* 1995, 1996, 1997; Meyer, 1991; Meyer *et al.,* 1992; Muthumeenakshi *et al.,* 1994; Zimand *et al.,* 1994).

1.3 Methodology

1.3.1 Isolation

Trichoderma species are readily isolated from soil by all available conventional methods, largely because of their rapid growth and abundant conidiation. Due to

the formation of chlamydospores and colonization of organic substrates, *Trichoderma* species are also readily obtained by soil-washing techniques. On wood, *Trichoderma* can frequently be observed as discrete colonies from which isolation into pure culture can be achieved in the field with moderate care. Similarly, the *Trichoderma* anamorphs of *Hypocrea* and related genera can be obtained from direct inoculations of ascospores or stromatal tissue.

To increase recovery, selective methods have been devised by Askew and Laing (1993), Davet (1979), Elad and Chet (1983), Elad *et al.* (1981), Johnson *et al.* (1987), Papavizas and Lumsden (1982) and Smith *et al.* (1990). Papavizas and Lumsden (1982) and Elad and Chet (1983) modified previous media (containing chloramphenicol, fenaminosulf, PCNB and rose bengal) by adding 20 mg/l captan after autoclaving. Smith *et al.* (1990) also added the detergent Igepal 630 (= nonoxynol 9) to Davet's medium. Askew and Laing (1993) replaced fenaminosulf by propamocarb or metalaxyl which were even better in suppressing oomycetes. For the selective isolation of *Gliocladium roseum* and *Trichoderma virens,* Park *et al.* (1992) used a medium containing benomyl, sodium propionate, rose bengal and antibacterial antibiotics supplemented with either 1 mg gliotoxin and 60 mg acriflavine, or 20 mg gliotoxin (per litre), respectively. Different temperature requirements displayed by certain species can be exploited for selective isolation (Johnson *et al.,* 1987; Komatsu, 1976; Widden and Hsu, 1987).

Composition of selective medium devised by Papavizas and Lumsden (1982):
Basal medium: V-8 juice—200 ml, water—800 ml; (g/l): agar—20; glucose—1; antimicrobial agents (μg/l): neomycin sulfate, bacitracin, penicillin G, and chloroneb—100; chlortetracycline hydrochloride—25, nystatin—20, sodium propionate—500; alkylaryl polyether alcohol is added to suppress Mucorales at 2.0 ml/l.
TSMC (Elad and Chet, 1983):
(g/l): $MgSO_4 \cdot 7H_2O$—0.2; K_2HPO_4—0.9; KCl—0.15; NH_4NO_3—1.0; glucose—3.0, chloramphenicol—0.25, fenaminosulf—0.3, pentachloronitrobenzene—0.2, rose bengal—0.15, captan—0.02 (post autoclaving), agar—20.
Modified TSM (Smith *et al.,* 1990):
(g/l): $Ca(NO_3)_2$—1.0, KNO_3—0.26, $MgSO_4 \cdot 7H_2O$—0.26, KH_2PO_4—0.12, $CaCl_2 \cdot 2H_2O$—1.0, citric acid—0.05, sucrose—2.0, agar—20.0, Igepal 630 (Alltech Associates, Inc., Deerfield, IL) —1.0 ml, chlortetracycline—0.05, captan (50% wettable powder) —0.04, vinclozolin—0.0025.

1.3.2 Cultivation

The methods described by Rifai (1969) are generally still followed. Good results can usually be obtained by point or streak inoculation on oatmeal, cornmeal or malt extract agars, and incubation under daylight for 5 or a few more days at a temperature of 20–25°C. Stable morphological features can be observed most easily on the first two media. On meagre media, such as potato-carrot agar or Nirenberg's SNA medium (Nirenberg, 1976), the phialides tend to be so thin-walled that they collapse within a short time and cannot be observed properly. The less rich Difco cornmeal agar seems to be a good intermediate to obtain reproducible results, but descriptions are not yet standardized for this medium. In the current study, cultures were incubated

for 2 days on malt extract agar in the dark, followed by incubation under ambient laboratory conditions of light and temperature (about 21°C). Linear growth was measured as the diameter of colonies after incubation in the dark for 4 days at 20 ± 1°C, from the point inoculation of conidia on 2% malt extract agar.

1.3.3 Observation

The production of conidia from effused conidiophores, or from conidiophores aggregated into fascicles or pustules, is observed in the undisturbed Petri dish using a dissecting or compound microscope. The sterile conidiophore extensions in sect. *Pachybasium* can be observed in the same fashion. Typical development of conidial aggregates is best observed on relatively dilute media, such as 2% malt extract. Diffusable pigments produced in the substrate are characteristic for some species. Characteristics of mycelial and pigment development are best observed on a relatively rich medium such as potato-dextrose agar.

For microscopic preparations, material should be taken from the growing colony margin—preferably young tufts where the conidia are just beginning to develop pigment. Conidiophore morphology and branching pattern, critical for identification to the sectional level, are best observed before conidia are completely mature, usually within 24 h of the onset of conidiation. Observations on conidial morphology and topology should be performed when conidia have matured—usually 7 to 14 days after the initial production of conidia. The material is mounted in lactic acid, with the optional addition of cotton blue. Examination under the oil immersion lens is essential to establish details of conidial shape and ornamentation.

1.4 Morphology

Rifai (1969) and Bissett (1991a) have discussed the morphological characters they used to characterize and differentiate species of *Trichoderma*. Both authors emphasized the difficulties inherent in defining morphological species of *Trichoderma*. Samuels (1996) also provided detailed observations and comments on the utility of morphological characters to define species in *Trichoderma*. Characters useful for characterization and identification in other Hyphomycete genera frequently are not as useful in differentiating *Trichoderma* species, usually because of the narrow range of variation of the simplified morphology in *Trichoderma,* or because descriptive terms to describe variation in colour or pattern are not sufficiently precise to define differences between species. Nonetheless, careful morphological observations are often sufficient for identification of species and strains of *Trichoderma,* at least to the extent that taxa have been adequately differentiated morphologically and described in the existing literature. Identifications based on morphological characters remain the primary method for identification and verification of species in *Trichoderma*.

Colony characters can be distinctive and characteristic of a species. However, colony appearance is difficult to describe with sufficient precision for it to be very useful for identification. Growth rates in culture can be useful to distinguish otherwise similar species. The production of conidia from effused conidiophores, or from conidiophores aggregated into fascicles or pustules is usually characterisitic of a species. Diffusable pigments can also be characteristic, although the colour

of such pigments does not vary a great deal in *Trichoderma*. Strains referable to section *Longibrachiatum* typically have conspicuous bright greenish-yellow pigments, at least when first isolated. Dull yellowish pigments are common in many species, but are not very distinctive. Some species are best characterized by a complete lack of pigment in reverse, whereas reddish pigments occur in reverse in a few isolates. Characteristic crystals produced in the media have been reported only for *Trichoderma aureoviride*. Indistinct mouldy or musty odours are commonly produced by different strains of *Trichoderma*. Characteristic aromatic odours resembling coconut are produced commonly by strains of *Trichoderma viride,* and sometimes also by *Trichoderma atroviride*.

The patterns of conidiophore branching and aggregation of conidiophores into fascicles and pustules are useful for identification of strains of *Trichoderma* to sections and species aggregates. Compact pustules are characteristic of many species in section *Pachybasium,* although of numerous strains in other sections as well. Conidiophore branching can be regularly verticillate or more irregular. Branches can be broad and straight or relatively narrow and flexuous. The conidiophore apex in some species in section *Pachybasium* characteristically ends in a sterile elongation which may be straight, undulate or coiled. Phialides can be disposed in regular verticils, or be paired, alternate or more irregularly disposed. Phialide shape is characteristic of the section; phialides are characteristically short and plump in section *Pachybasium,* whereas in section *Longibrachiatum* they are elongate and lageniform to nearly cylindrical. Terminal phialides in most species tend to be more elongate and narrower and frequently more or less subulate. Subterminal cells of the conidiophore may produce conidia through a short lateral neck, thus intercalary phialides or what Gams (1971) called aphanophialides; these are rather commonly seen in *Trichoderma* sect. *Longibrachiatum*. Conidial shape varies from globose, to ellipsoidal, obovoidal, or short-cylindrical, with the basal end more or less tapering and truncate. The overall range of variation in conidial dimensions in *Trichoderma* is not great, however related species can often be differentiated by slight but consistent differences in size. The conidial surface appears smooth in most species in light microscope observations, although many species with apparently smooth conidia are delicately ornamented when examined by SEM. Conidia can also be variously roughened or verrucose in the *T. viride* aggregate, and conidia can have wing-like or bullate projections of the outer wall in two species—*T. saturnisporum* and *T. ghanense*. Conidial pigments are also characteristic, varying from colourless (white in mass), to various green shades, or less often grey or brown. In some species mature conidia appear dark green in the microscopic mount, in others only pale. Chlamydospores are common in many species, although they tend to be uniformly globose or ellipsoidal, terminal and intercalary, smooth-walled, colourless, yellowish or greenish, and 6–15 μm diameter in most species. Vegetative hyphae show few characters useful for identification.

1.4.1 Delimitation of the genus Trichoderma

Trichoderma strains can often be readily identified to genus by a distinctive morphology that includes rapid growth, bright green or white conidial pigments, and a repetitively branched, but otherwise poorly defined conidiophore structure. Bissett (1991a) proposed including all the anamorphs of *Hypocrea* in the genus *Trichoderma*. If

this concept is accepted, then a clear morphological definition for the genus *Trichoderma* would be problematic (for discussion see Samuels, 1996) since the conidiophore branching structure is highly variable, and in many cases superficially resembles unrelated genera such as *Verticillium* and *Gliocladium*. In most of these cases, the *Trichoderma* anamorphs of *Hypocrea* can be differentiated in having a less regular pattern of branching at an indefinite number of levels—as opposed to the more regular verticillate or biverticillate branching in *Verticillium;* and the production of a terminal penicillate arrangement of branches and more regularly aculeate phialides on a relatively well-defined stipe in *Gliocladium*. However, the anamorph of *Hypocrea lutea* (Tode: Fr.) Petch (which resembles *Gliocladium viride* Matr.) and *H. gelatinosa,* for example, tend to have a penicillate conidiophore structure that is more characteristic of the genus *Gliocladium*. The convergent phialides of *Trichoderma (Gliocladium) virens* in otherwise *Trichoderma*-like conidiophores cannot be taken as an argument for excluding this species from *Trichoderma.*

Samuels (1996) hypothesized that the anamorphs of species of *Hypocrea* with effused stromata having colourless conidia on irregularly verticillate conidiophores, and placed by Bissett (1991a) in *Trichoderma* section *Hypocreanum,* may be synanamorphs or spermatial states, and therefore inappropriately placed in *Trichoderma*. This possibility is evidenced in *Hypocrea sulawesensis* Doi and *H. poronioidea* A.S.Möller (Samuels and Lodge, 1996) by the production of hyaline conidia produced terminally on sparingly branched conidiophores—in addition to a typical *Trichoderma* anamorph. In any case, section *Hypocreanum* is clearly polyphyletic and may well prove to be an untenable grouping as currently defined. The relationships of species that might be accommodated in section *Hypocreanum* can probably only be determined by resolution of their teleomorph connections and genetic relationships through macromolecular investigations. These species are rarely encountered other than as ascospore isolations from the teleomorph, and they are not dealt with further here. The generic description that follows accommodates the full range of variation evident in clonal strains of *Trichoderma* and anamorphs of *Hypocrea* encountered independently of the teleomorph state.

1.5 The genus *Trichoderma*

Trichoderma Pers.: Fr.

Trichoderma Persoon, Römer's Neues Mag. Bot. 1:92. 1794: Fries, Syst. mycol. 1: xlv. 1821 and Syst. mycol. 3:214. 1829.

SYNONYMY, see Rifai (1969).
LECTOTYPE SPECIES: *T. lignorum* (Tode) Harz *fide* Clements and Shear (1931), non-sanctioned name, which refers to the same fungus as *Trichoderma viride* Pers.: Fr. designated by Hughes (1958).

Colonies usually growing rapidly, mycelium initially submerged, eventually with variably matted, floccose, woolly or arachnoid hyaline aerial mycelium, depending on the strain and culture medium. Reverse uncoloured or variously buff, yellow, amber, dull reddish or yellow-green. Odour mostly pronounced or faint, characteristic of the genus, suggesting coconut or camphor. **Conidiation** effuse or tufted or forming compact pustules; typically in green shades, or less often white, grey or brown. **Conidiophores** in most species with a broad main axis branched at regular intervals, usually with successive branches apically and distally progressively shorter and narrower;

branches more or less divergent, solitary, paired or in verticils; repetitive verticillate branching may result in a highly ramified pyramidal structure; in other species branching is less regular with branches solitary or paired and not extensively rebranched. In section *Pachybasium,* the conidiophore main axis and primary branches are often terminated by sterile conidiophore elongations which may be simple or branched, straight, flexuous, undulate, hamate or coiled; anastomoses may occur between adjacent conidiophores. Frequently phialides and fertile branches also arise from otherwise undifferentiated aerial hyphae in areas of effuse conidiation. **Conidiogenous cells** phialides, typically disposed in divergent verticils terminally on branches of the conidiophore, or in whorls directly beneath septa along the conidiophore and branches, otherwise paired or solitary and irregularly disposed; cylindrical, subulate, lageniform, ampulliform or subglobose; usually attenuated to a narrow, short-cylindrical, conidium-bearing neck. **Conidia** one-celled, typically green, or otherwise colourless, greyish, or brownish; smooth-walled to distinctly roughened, or with sinuate, bullate or wing-like projections from the outer wall; subglobose, obovoid, ellipsoid, oblong or short-cylindrical; accumulating in gloeoid heads, sometimes enclosed in a sac-like sheath visible at high magnifications (SEM). **Chlamydospores** usually present and often abundant, especially in the submerged mycelium; intercalary, or terminal on short lateral branches of vegetative hyphae; globose to ellipsoid, colourless to pale yellowish or greenish, smooth-and sometimes thick-walled (to 4 μm). **Vegetative hyphae** usually hyaline, smooth-walled, (1–)2–10 μm wide, less often (or in the submerged mycelium) pale yellow, with irregular wall thickenings and up to 16 μm wide. Teleomorph, where known, *Hypocrea, Podostroma* or *Sarawakus*.

Infrageneric classification of *Trichoderma*. —Rifai (1969) divided the genus into nine species aggregates defined largely by conidiophore morphology. Rifai's system is still employed for identification, although it does not accommodate the full range of morphological variation that is currently recognized. Bissett (1991a) proposed a revised classification, in large part based on the species aggregates of Rifai, that divided the genus into five sections, also defined by characteristics of the conidiophore branching system. This new classification was proposed to relate morphologically similar forms, and also to accommodate the wider range of morphological variation expressed by anamorphs of *Hypocrea*. Bissett expanded the concept of *Trichoderma* to also include some forms previously accepted in *Gliocladium,* although recognizing that the sections were almost certainly not monophyletic. Apart from the need to merge sect. *Saturnisporum* Doi *et al.* (1987) with sect. *Longibrachiatum,* it appears premature to propose an improved classification for *Trichoderma,* and the identification scheme outlined below employs the classification into four sections similar to that proposed by Bissett (1991a).

1.5.1 Keys

Key to the sections of *Trichoderma*

1(a) Conidiation effuse, conidia not green; conidiophores with few or no lateral branches; phialides borne in simple terminal verticils, cylindrical to lageniform, *"Cephalosporium"*-like . . . . section *Hypocreanum*

1(b) Conidiation effuse or fasciculate to pustulate, conidia often green; conidiophores with frequent lateral branches; phialides mostly lageniform to ampulliform 2

2(a) Conidiophore main axes long with short secondary branches, not extensively rebranching; branches and phialides frequently arising singly, particularly the terminal ones; conidia smooth but sometimes with conspicuous, sinuate, wing-like or bullate ornamentation . . . . section *Longibrachiatum*
2(b) Conidiophores repeatedly rebranching; branches and phialides paired or verticillate, also those in terminal position; conidia smooth-walled or verrucose 3
3(a) Conidiophores and branches relatively broad (main axis to 10 μm wide); phialides in verticils of 2–7, ampulliform to lageniform; conidia green, brownish or hyaline . . . . section *Pachybasium*
3(b) Conidiophores and branches narrow and flexuous (main axis to 6 μm wide); phialides mostly in verticils of 2 or 3(–5), lageniform to subulate; conidia always green . . . . section *Trichoderma*

The species in section *Trichoderma* have narrow and flexuous conidiophores; with branches and phialides uncrowded, frequently paired, and seldom in verticils of more than three. Section *Pachybasium* (Sacc.) Bissett includes species with highly ramified, broad conidiophores usually arranged in compact pustules or fascicles, and with branches and phialides broad or inflated, relatively short, and disposed in crowded verticils. Some species are further characterized by the production of sterile conidiophore extensions, and many isolates produce compact conidiogenous pustules with adjacent conidiophores often anastomosing. In section *Longibrachiatum* Bissett (1984) the conidiophores are sparingly and irregularly branched, with the phialides also irregularly disposed and not usually in whorls or verticils. Species in section *Longibrachiatum* often produce distinctive greenish-yellow pigments in the reverse of cultures. Section *Saturnisporum* Doi *et al.* (1987) was characterized by a branching system similar to section *Longibrachiatum,* with branches and phialides uncrowded and frequently paired, but with inflated phialides and compact conidiogenous pustules as in section *Pachybasium*. The section was further differentiated by the bullate or wing-like conidial ornamentation. Molecular data (Kuhls *et al.,* 1997) have shown that its species are intimately related to others in sect. *Longibrachiatum* and the branching patterns of the conidiophores also fit this section. Section *Hypocreanum* Bissett (1991a) accommodates *Hypocrea* anamorphs, mostly from section *Homalocrea* (Sacc.) Doi, and occasional isolates from soil or wood that are characterized by effuse, usually sparse conidiation, sparingly branched conidiophores, and cylindrical to subulate phialides frequently borne in *Verticillium*-like divergent verticils. Old or stale cultures of species in the other sections may develop a similar morphology in effuse areas of conidiation.

The following keys serve to identify most known *Trichoderma* species, although not all *Trichoderma* anamorphs of *Hypocrea* and related genera known have been included. In particular, the *Hypocrea* anamorphs referable to *Trichoderma* section *Hypocreanum,* which are rarely observed independently of the teleomorph, are not treated. Neither are all known or suspected teleomorph connections applied to species aggregates cited here—their morphological distinctions *in vitro* require much further study.

Key to species in *Trichoderma* section *Trichoderma*

1(a) Colonies slow-growing, reaching less than 6 cm diam. in 7 days at 20°C 2
1(b) Colonies faster growing 3

2(a) Colonies yellow-green, floccose; phialides slender, almost *Verticillium*-like but curved; conidia obovoid with truncate base, 3.0–5.0 × 2.0–3.2 μm . . . . 2. *Trichoderma aureoviride* anam. of *Hypocrea aureoviridis*
2(b) Colonies green, granular, tardily sporulating; phialides flask-shaped; conidia ellipsoid, 3.5–5.0 × 2.0–2.6 μm . [*Trichoderma* anam. of *Podostroma alutaceum*]
3(a) Conidia cylindrical, 3.0–4.8 × 1.9–2.8 μm . . . . 4. *T. koningii* aggregate
3(b) Conidia subglobose to short-ellipsoidal or ovate 4
4(a) Conidia ovate with truncate base, 3.5–5.0 × 2.5–3.2 μm . . 5. *Hypocrea vinosa*
4(b) Conidia subglobose to short-ellipsoidal 5
5(a) Fully mature conidia pale green, 2.5–3.5 × 2.1–3.0 μm . . . . 3. *T. harzianum* aggregate
5(b) Fully mature conidia becoming dark green 6
6(a) Fully mature conidia more or less roughened, 3.6–4.5 μm diam. or 4.0 4.8 × 3.5–4.0 μm . . . . 6. *T. viride* aggregate
6(b) Fully mature conidia smooth-walled, usually 2.6–3.8 × 2.2–3.4 μm . . . . 1. *T. atroviride*

Key to species in *Trichoderma* section *Pachybasium*

(modified from Bissett, 1991b)

1(a) Conidiation entirely effuse, or conidiophores arranged in loosely organized flat pustules or small irregular fascicles; conidiophores sparingly branched with principal branches most often arising singly or paired [if conidia are hyaline, see 9(b)] 2
1(b) Conidiophores organized in compact, hemispherical to cushion-shaped pustules; conidiophores usually highly branched with branches 2–4-verticillate . . . 8
2(a) Conidiophores arranged in fascicles up to 2 mm diam. 3
2(b) Conidiophores effuse, or loosely arranged in flat pustules 4
3(a) Colonies less than 4 cm diam. after 4 days at 20°C; aerial hyphae more than 1.5 μm wide; phialides convergent; chlamydospores infrequent; conidia 2.5 4.5 × 2.0–3.1 μm . . . . 12. *Trichoderma* anam. of *Hypocrea gelatinosa*
3(b) Colonies more than 4 cm diam. after 4 days; aerial hyphae mostly less than 1.5 μm wide; phialides divergent; chlamydospores abundant in older mycelium; conidia 2.9–4.0 × 2.0–2.9 μm . . . . 9. *T. fasciculatum*
4(a) Conidia subglobose to obovoid, smaller than 3.5 × 2.5 μm, rather pale green 5
4(b) Conidia broadly ellipsoidal, larger than 3.5 × 2.5 μm, of other colour 6
5(a) Conidiation usually diffuse; phialides flask-shaped, 5.5–7.5 (in terminal position–10) × 2.5–3.2 μm, in divergent clusters of 3–5; conidia subglobose to short-obovoid, 2.5–3.5 × 2.1–3.0 μm . . . . see 3. *T. harzianum* aggregate
5(b) Conidiation more or less pustular but lacking sterile appendages; phialides broadly flask-shaped, 4–5(–7) × 2.3–3.0 μm; conidia subglobose, 2.3– 3.0 × 2.0–2.6 μm . . . . 14. *T. inhamatum*
6(a) Conidia pale brown, 3.0–5.3 × 2.4–4.3 μm . . . . 11. *T. flavofuscum*
6(b) Conidia dark green 7
7(a) Conidiophores aggregated into flat pustules on MA, usually with sterile apical elongations; phialides divergent; conidia 3.7–5.3 × 2.6–3.7 μm . 7. *T. crassum*
7(b) Conidiation entirely effuse, or conidiophores lacking sterile apical elongations; phialides convergent in penicillate manner; conidia 3.5–6.0 × 2.8–4.1 μm . . . . 26. *T. virens*

8(a) (1) Conidiation white to buff 9
8(b) Conidiation eventually green to grey 10
9(a) Conidiophores with spiral, sterile apical elongations; phialides divergent; conidia ellipsoidal, 2.3–3.6 × 1.4–2.2 μm . . . . 19. *T. polysporum*
9(b) Conidiophores lacking sterile elongations; phialides more or less convergent; conidia subglobose, 2.5–3.5 μm diam. . . . . 18. *T. piluliferum*
10(a) Conidiophores with conspicuously roughened, spiral, sterile apical elongations; conidiation bright greenish-yellow or rosy-buff; conidia 2.8– 4.0 × 1.8–2.5 μm . . . . 8. *T. croceum*
10(b) Conidiophores lacking sterile elongations or elongations not roughened; conidiation in various green or grey shades 11
11(a) Conidiophores arranged in pustules up to 2 mm diam., glaucous to greyish; and conidiophore main axis 4.5–7 μm wide over the fertile part; conidia 3.0–4.6 × 1.8–2.7 μm . . . . 21. *Trichoderma* anam. of *Hypocrea semiorbis*
11(b) Conidiophore pustules larger, usually in definite green shades; or conidiophore main axis not exceeding 5.5 μm wide over the fertile part 12
12(a) Conidia consistently less than 3.5 μm long and 2.5 μm wide 13
12(b) Conidia mostly longer and/or wider 15
13(a) Conidia subglobose to broadly obovoid . . . . see 5
13(b) Conidia ellipsoidal, 2.5–3.4 × 1.8–2.3 μm 14
14(a) Conidiogenous areas bright green to yellow-green, conidiophore main axis branched and fertile to apex . . . . 16. *T. minutisporum*
14(b) Conidiogenous areas grey-green, conidiophore main axis with conspicuous spiral sterile apical elongations . . . . 25. *T. tomentosum*
15(a) Colony reverse conspicuously in pigmented yellow to reddish-brown shades; conidiophore main axis very stout, 4–6.5 μm wide at base of sterile elongation 16
15(b) Colony reverse colourless to pale dull yellowish; conidiophore main axis usually 3.5–5 μm wide at base of sterile elongation 17
16(a) Conidiophore main axis relatively straight throughout, the upper part unbranched and nonfertile to near the apex, which is terminated by a single phialide or more often by 2–3 short fertile branches; conidia 3.0–4.5 × 1.9–2.5 μm . . . . 10. *T. fertile*
16(b) Conidiophore main axis with a spiral, sterile apical elongation, never with fertile branches near the apex; conidia 3.0–4.4 × 1.8–2.7 μm. .. 22. *T. spirale*
17(a) Conidia strictly cylindrical, frequently longer than 4.5 μm, never shorter than 3.5 μm 18
17(b) Conidia cylindrical to ellipsoid, rarely longer than 4.5 μm, often shorter than 3.5 μm 19
18(a) Conidiophore main axis with undulate to spiral sterile elongation which is highly branched and anastomosing to within 100 μm of the acute apex; conidia 3.9–5.7 × 2.0–3.0 μm .15. *T. longipile*
18(b) Conidiophore main axis with a straight to flexuous sterile elongation which is sparingly branched with a bluntly rounded apex; conidia 3.5–5.0 × 1.7–2.8 μm . . . . 17. *T. oblongisporum*
[if conidia broadly ellipsoidal, 3.7–5.3 × 2.6–3.7 μm, see also 7(a)]
19(a) Conidiophore main axis with straight to flexuous sterile elongation 20
19(b) Conidiophore main axis with undulate to coiled or circinate, sterile elongation 21

20(a) Conidiogenous pustules bluish green, appearing spiny due to the presence of stiff, javelin-like sterile conidiophore apices; conidia 3.0–4.8 × 1.8–2.5 μm . . . . 24. *T. strigosum*

20(b) Conidiogenous pustules dull-green, appearing hairy due to presence of very long, straight or flexuous sterile conidiophore apices; conidia 2.8–4.0 × 2.2– 3.0 μm . . . . 23. *T. strictipile*

21(a) Conidiogenous pustules bluish-green 22

21(b) Conidiogenous pustules bright-green, surface appearing downy due to the presence of branched, undulate, thin, sterile conidiophore apices; conidia 3.1– 4.7 × 2.0–2.9 μm . . . . 20. *T. pubescens*

22(a) Conidiogenous pustules appearing velvety due to presence of strongly undulate or hamate, sterile conidiophore apices; conidia 3.0–4.5 × 2.1–2.8 μm . . . . 13. *T. hamatum*

22(b) Conidiogenous pustules appearing woolly due to presence of coarse, spiral conidiophore apices; conidia 3.0–4.4 × 1.8–2.7 μm . . . . 22. *T. spirale*

Key to species in *Trichoderma* section *Longibrachiatum*

1(a) Conidia ellipsoid, ornamented with conspicuous wing- and wart-like ornamentation, 3.6–5.8 × 2.8–3.4 μm, dark green . . . . 33. *T. saturnisporum*

1(b) Conidia generally smooth-walled or some conidia with pustulate extensions of the outer layer of the wall 2

2(a) Conidiophores bearing side branches that are commonly rebranched once or twice; phialides distinctly constricted at the base; conidia mostly smaller than 4.0 × 2.5 μm 3

2(b) Conidiophores very sparingly branched; phialides nearly cylindrical and hardly or not at all constricted at the base; conidia mostly larger than 4.0 × 2.5 μm . 4

3(a) Colonies with conidial areas typically forming large compact pustules; conidiation in yellow-green shades or dark olive in older cultures; conidia ellipsoidal and mostly smaller than 3.5 × 2.0 μm . . . . .27. *T. citrinoviride*

3(b) Colonies with conidial areas widely effused and not forming pustules; conidiation mostly in bluish-green shades and not darkening appreciably in age; conidia ellipsoid to nearly cylindrical and mostly 4.0–5.0(–6.0) × 2.5–3.0 μm . . . . 30. *T. pseudokoningii*

4(a) Conidia mostly longer than 5.0 μm, usually measuring 6.0–7.5 × 3.0–3.7 μm, ellipsoidal, dark green, often mixed with narrower, paler, cylindrical conidia, 5.0–6.0 × 2.0–2.5 μm 5

4(b) Conidia usually shorter than 5.0 μm 6

5(a) Conidia consistently smooth-walled . . . . 29. *T. parceramosum*

5(b) Conidia narrow oval to subcylindrical, 5.6–8.2 × 2.9–3.8 μm, most smooth-walled, but some with pustulate extensions of the outer layer of the conidial wall . . . . 32. *T. ghanense*

6(a) Conidia short-cylindrical to obovoid, with a conically tapering base, 4.0–5.5 × 2.0–2.5 μm; reverse uncoloured . . . . 28. *T. longibrachiatum*

6(b) Conidia obovoid to ellipsoid, 3.5–4.5 × 2.3–3.0 μm; colony exuding yellow pigment into the agar . . . . 31. *T. reesei*

1.5.2 Species descriptions

Species descriptions are from observations on 2% malt extract, unless otherwise specified. Cultures were incubated for 2 days in the dark, followed by incubation

under ambient laboratory conditions of light and temperature (about 21°C). Linear growth was measured as the diameter of colonies, inoculated from conidia, after 4 days at 20 ± 1°C on 2% malt extract agar.

Trichoderma* section *Trichoderma

Colonies growing slowly or rapidly depending on the species, aerial mycelium usually limited, floccose to arachnoid; reverse colourless to dull yellowish. Some isolates with a distinctive aromatic odour resembling coconut. **Conidiation** variably effuse, loosely tufted, or forming compact pustules; white at first, eventually green (rarely brown). **Chlamydospores** present in most isolates, frequently abundant. **Conidiophores** usually relatively narrow and flexuous; with primary branches arising at regular intervals, usually paired or in whorls of three, usually short and not extensively rebranched. **Phialides** mostly in verticils of 2 or 3, in some strains up to 5-verticillate, lageniform to subulate. **Conidia** green (rarely brownish), smooth-walled to distinctly verrucose, subglobose to obovoid or ellipsoid.

Type species: *Trichoderma viride* Pers.: Fr.

1. *Trichoderma atroviride* P.Karsten, Finl. Mögelsvamper p. 21 = Bidr. Känned. Finl. Natur Folk 51:363. 1892. (Fig. 1.2).

[non *Trichoderma atroviride* Bissett, Can. J. Bot. 62:930. 1984, nom. illeg. Art. 53 = *Trichoderma parceramosum* Bissett, Can. J. Bot. 69:2418. 1991].

DESCRIPTION: Bissett, Can. J. Bot. 70:639. 1992.

Colonies growing rapidly (5–8 cm). **Conidiation** appearing granular or crusty in age; initially glaucous, rapidly turning dark green. Reverse usually uncoloured, otherwise dull yellowish or drab in age. Odour often pleasantly aromatic resembling coconut. **Conidiophores** characterisitic of the section. **Phialides** solitary, or 2–4-verticillate, more or less lageniform, often curved, 6–12 × 2.4–3.0 μm. **Conidia** dark green, smooth (even under SEM), subglobose at maturity, 2.6–3.8(–4.2) × 2.2–3.4(–3.8) μm.

2. *Trichoderma aureoviride* Rifai, Mycol. Pap. 116:34. 1969. (Fig. 1.5).

TELEOMORPH: *Hypocrea aureoviridis* Plowr. & Cooke, in Phill. & Plowr., Grevillea 8:104. 1880.

DESCRIPTION: Rifai and Webster, Trans. Br. Mycol. Soc. 49:289–296. 1969.

Colonies slow-growing (1–2.5 cm). **Conidiation** forming compact dull green tufts or pustules. Colony reverse conspicuously discoloured brownish-yellow, in part due to the production of yellow crystals. **Conidiophores** as in the section, except more regularly verticillate and repeatedly branched toward the base, forming a pyramidal structure. **Phialides** usually 2- or 3-verticillate, narrow ampulliform or lageniform, 7–18 × 2–2.5 μm. **Conidia** pale green, obovoid, with truncate base, smooth, 3.0– 5.0 × 2.0–3.2 μm.

3. *Trichoderma harzianum* Rifai, Mycol. Pap. 116:38. 1969. (Fig. 1.3).

DESCRIPTION: Gams and Meyer, Mycologia 90 (in press). 1998.

Colonies growing rapidly (most isolates 7–9 cm). **Conidiation** predominantly effuse, appearing granular or powdery due to dense conidiation; rapidly turning yellowish-green to dark green, or producing tufts or pustules fringed by sterile white mycelium. Reverse colourless to dull yellowish, buff or drab. Odour indistinct or faintly earthy. **Conidiophores** as in the section, tending to be regularly verticillate

forming a pyramidal structure. **Phialides** ampulliform to lageniform, usually 3–4-verticillate, occasionally paired, mostly 3.5–7.5 × 2.5–3.8 μm, terminal phialides up to 10 μm long. **Conidia** subglobose to obovoid, mostly (2.5–)2.7–3.5 × 2.1–2.6(–3.0) μm, smooth-walled, subhyaline to pale green.

This species, even in the strict sense (Gams and Meyer, 1998) comprises the strains often used in biological control of plant-pathogenic fungi (see Volume 2, Chapter 6). Its temperature optimum for growth is at 30°C with a daily increment of 3.3 cm on malt agar, maximum growth temperature is 36°C. The taxon differs from *T. inhamatum,* which has a stronger tendency to pustular sporulation, broader phialides, and slightly smaller conidia (Veerkamp and Gams, 1983). Morphologically, these two taxa seem to take an intermediate position between the sections *Trichoderma* and *Pachybasium*. Aggressive weed moulds in commercial mushroom cultivation are usually classified in this species aggregate, as Th2 for British isolates (Doyle, 1991; Seaby, 1987) and Th4 for North American isolates (Seaby, 1996). Th2 sporulates in small tufts, has slightly smaller conidia, 2.5–3.0(–3.3) × 2.3–2.8 μm, its temperature optimum is also 30°C, but with a daily increment of 1.8–2.2 cm, while at 36°C no growth occurs. Th4 shows more abundant sporulation in compact aggregations of the copious aerial mycelium and conidia decolouring in lactophenol (Seaby, 1996). *T. atroviride* (q.v.) has darker and somewhat larger conidia and the colonies often have a more pronounced odour suggesting coconut.

4. *Trichoderma koningii* Oud., in Oudemans and Koning, Arch, néerl. Sci. II, 7:291. 1902. (Fig. 1.6).

= *Acrostalagmus koningii* (Oud.) Duché & Heim, Trav. Crypt, déd. Louis Mangin, p. 440. 1931.

Colonies usually fast-growing (7–9 cm). **Conidiation** occasionally forming compact tufts or eventually concrescent, forming continuous crusts, dull green to bluish-green. Reverse usually colourless, less often pale yellowish. **Conidiophores** variable, in some strains narrow and flexuous with elements mostly paired, or in others broader and more rigid with elements verticillate. **Phialides** lageniform or more or less ampulliform, usually paired or irregularly disposed in strains with flexuous conidiophores, or 3–5-verticillate in strains with relatively broad conidiophores, mostly 7.5–12 × 2.5–3.5 μm, or terminal phialides more elongate. **Conidia** subcylindrical to narrow ellipsoid, green, 3.0–5.5 × 1.9–3.2 μm.

Isolates from the type locality representing *T. koningii* s. str. show a characteristic diffuse sporulation on cobwebby aerial mycelium in addition to the conidial tufts; the conidia measure 3.7–4.5 × 2.0–2.5 μm. The same isolates have a temperature optimum of 24°C, and a maximum near 33°C (Lieckfeldt *et al.* 1998).

5. *Trichoderma* sp. anamorph of *Hypocrea vinosa* Cooke, Grevillea 8:65. 1879.

DESCRIPTIONS: Dingley, Trans. R. Soc. N. Z. 79:330–331. 1952, and 84:693. 1957; Rifai and Webster, Trans. Br. Mycol. Soc. 49:290. 1966.

Colonies fast-growing (7–9 cm). **Conidiation** effuse or loosely fasciculate, yellowish-green to olivaceous-green. Colony reverse pale or dull yellowish. **Conidiophores** regularly verticillate and repeatedly branched toward the base, forming a pyramidal structure. **Phialides** more or less ampulliform, mostly 6– 10 × 2.5–3.5 μm. **Conidia** pale green, obovoid, smooth, 3.5–5.0 × 2.5–3.2 μm.

6. *Trichoderma viride* Pers., Römer's neues Mag. Bot. 1:92. 1794. Fries, Syst. mycol. 3:215. 1829. (Fig. 1.1).

= *T. lignorum* (Tode) Harz; for other synonymy see Rifai (1969).

TELEOMORPH: Hypocrea rufa (Pers.: Fr.) Fr.

DESCRIPTIONS: Webster (1964), Rifai (1969), Meyer and Plaskowitz (1989).

Colonies fast-growing (5–9 cm). **Conidiation** forming compact tufts or more effuse, glaucous to dark bluish-green. Reverse typically uncoloured, less often pale yellowish. Odour usually distinctly aromatic, as of coconut. **Conidiophores** usually not extensively branched and having a relatively loose arrangement, branches most often paired, or single or 3-verticillate, often appearing flexuous. **Phialides** frequently paired, or arising singly or 3-verticillate, narrowly lageniform, 8–14 × 2.4–3.0 μm. **Conidia** globose to ellipsoidal, usually conspicuously warted, bluish-green to dark green, 4.0–4.8 × 3.5–4.0 μm.

The above description is for ascospore isolates of *Hypocrea rufa. Trichoderma* isolates with ornamented conidia were all placed by Rifai (1969) in the *T. viride* aggregate. There has been no further taxonomic resolution of strains in the *T. viride* aggregate, although significant morphological variation is evident. Conidial shape varies from globose to ellipsoid or oblong. Some strains have flexuous conidiophores and regularly paired branches and phialides. Other isolates have conidiophores broader and appearing more rigid, with phialides shorter and frequently paired but also more irregularly disposed. Significant variation in the morphology of conidial ornamentations is also reported by Rifai (1969). While the anamorph of *H. rufa* has coarsely warted conidia, Meyer and Plaskowitz (1989) described a second group with fine irregularly pyramidal warts in SEM.

Trichoderma* section *Pachybasium (Sacc.) Bissett, Can. J. Bot. 69:2362. 1991.

= *Pachybasium* Sacc., Rev. Mycol. Toulouse 7:160. 1885.

For descriptions see Bissett (1991b).

Colonies slow to fast-growing, variable among species (2–9 cm), reverse colourless to yellow, amber or reddish. **Conidiation** effuse, tufted, or more characteristically aggregated into compact fascicles or pustules; white to grey, green or brown. **Conidiophores** often aggregated in fascicles or pustules, complexly and repetitively branched at regular intervals, with branches often crowded, relatively broad, usually increasing in length to the base of the conidiophore and rebranched several times. The conidiophore apex in many species is characteristically extended into sterile appendages, unbranched or branched, and infertile near the apex, sometimes anastomosing with adjacent conidiophore tips. **Phialides** in verticils of 2–7, often crowded, relatively short and wide, ampulliform to lageniform, except in terminal positions or in effuse areas of conidiation and then occasionally lageniform to subulate. **Conidia** colourless to grey, green or brown, appearing smooth-walled in the light microscope (some species with conidia delicately roughened in SEM observations), ellipsoid to obovoid or subglobose. In effuse areas of conidiation, micronematous conidiophores arising from the aerial mycelium are less complexly branched than described above, lack sterile apices, and have relatively narrow or cylindrical branches, with phialides often more elongate and convergent.

Type species: *Trichoderma hamatum* (Bon.) Bain. (basionym: *Verticillium hamatum* Bonorden, Handb. allg. Mykol. p. 97. 1851).

7. *Trichoderma crassum* Bissett, Can. J. Bot. 69:2376. 1991.

Colonies growing rapidly (5–6.5 cm). **Conidiation** effuse, or aggregated in compact, flat pustules, dull green, or quickly turning dark green. Reverse colourless or slowly turning dull yellowish to amber. Odour indistinct. **Conidiophores** coarse, erect,

branching irregularly, primary branches arising singly or 2–3-verticillate, rebranched once or twice; the apical part of the main conidiophore axis straight or slightly flexuous, sterile and unbranched for up to 150 μm to the apex; in areas of effuse conidiation phialides arising singly or in whorls of 2–4 on prostrate, undifferentiated hyphae, or on short, lateral branches from the aerial mycelium. **Phialides** from macronematous conidiophores ampulliform, 4.4–9.5 × 3.0–4.2 μm, arising in crowded whorls of 2–5 terminally or laterally on the conidiophore and its branches, usually reflexed toward the apex of the conidiophore and appressed rather than divergent; phialides from undifferentiated hyphae in areas of effuse conidiation ampulliform to lageniform or subulate, up to 30 μm long × 2.5–4 μm wide. **Conidia** broadly ellipsoidal, 3.7–5.3 × 2.6–3.7 μm, smooth-walled, dark green.

8. *Trichoderma croceum* Bissett, Can. J. Bot. 69:2379. 1991.

Colonies growing moderately slowly (3–4 cm). **Conidiation** typically in compact pustules, or concrescent to form irregular or concentric masses; white at first and slowly turning bright greenish-yellow or pale rosy-buff. Reverse colourless or buff. Odour indistinct. **Conidiophores** branched as typical of the section; the conidiophore apex loosely spiral-shaped, nonfertile and unbranched, and at maturity conspicuously roughened or tuberculate. **Phialides** ampulliform, mostly 4.0–6.8 × 2.6–3.8 μm, 2–5-verticillate, but mostly in uncrowded verticils of 2 or 3. **Conidia** broadly ellipsoidal, 2.8–4.0 × 1.8–2.5 μm; smooth-walled, subhyaline viewed singly, greenish-yellow in mass.

9. *Trichoderma fasciculatum* Bissett, Can. J. Bot. 69:2379. 1991.

Colonies growing moderately rapidly (4–5.5 cm). **Conidiation** slow to develop, after about 10 days forming small, irregular, evenly distributed fascicles, slowly turning dull green. Reverse colourless or pale buff to yellowish. Odour lacking or indistinct. **Conidiophores** branching irregularly and somewhat sparingly, primary branches relatively long, arising singly or more often in pairs or groups of three, rebranched irregularly; the conidiophore main axis usually with a narrow, flexuous, unbranched, nonfertile part below the apex, the apical cell usually bearing a single, subulate phialide, or sometimes a terminal verticil of lageniform phialides. **Phialides** mostly lageniform to ampulliform, 4.2–7.8 × 2.2–3.5 μm, arising singly or 2–3(–5)-verticillate (usually 2–3). **Conidia** broadly ellipsoidal, mostly 2.9–4.0 × 2.0–2.9 μm, very rarely up to 6 × 3 μm; smooth-walled, greenish.

10. *Trichoderma fertile* Bissett, Can. J. Bot. 69:2382. 1991. (Fig. 1.13).

Colonies growing moderately rapidly (4–6 cm). **Conidiation** forming flat or cushion-shaped pustules, quickly shading to characteristic yellow-green shades, occasionally fringed with a narrow band of white mycelium. Reverse conspicuously pigmented yellow to greenish-yellow. Odour faintly "mouldy". **Conidiophores** highly branched but with branches short giving the conidiophore a linear appearance; primary branches in whorls of 2 or 3; frequently rebranched, with secondary branches short (1- or 2-celled); the upper part of the conidiophore appearing stiff and relatively straight, and nonfertile and unbranched for about 60–120 μm above the fertile part; apex always fertile at maturity and terminated by a single apical phialide, or more often by 2 or 3 simple, short, fertile branches or combinations of branches and lateral phialides. **Phialides** mostly ampulliform, 3.2–7.0 × 2.4–3.8 μm, usually arising in whorls of 2–5, less often singly. **Conidia** ellipsoidal to oblong mostly 3.0–4.5 × 1.9–2.5 μm, smooth-walled, medium green.

11. *Trichoderma flavofuscum* (J.Miller, Giddens & Foster) Bissett, Can. J. Bot. 69: 2385. 1991.

= *Gliocladium flavofuscum* J.Miller, Giddens & Foster, Mycologia 49:793. 1957.

Colonies growing rapidly (6–7.5 cm). **Conidiation** mostly effuse and covering the plate; occasionally forming fascicles or flat pustules; very quickly turning yellowish-brown. Reverse colourless or slowly developing dull yellowish or drab shades. Odour indistinct. **Conidiophores** from effuse areas of conidiation arising from aerial hyphae, the basal part sparingly branched, at the apex with a cluster of 3–5 phialides, or a mixture of branches and phialides, arising at acute angles and appressed toward the apex; conidiophores from pustules more complexly branched, with branches short, mostly 1- or 2-celled. **Phialides** ampulliform to lageniform, mostly 5–10 × 2.6–5.0 μm, or solitary phialides more elongate, arising usually in terminal clusters of 2–5, rarely arising laterally on the conidiophore or its branches, often crowded with tips convergent and conidia from adjacent phialides coalescent into large heads. **Conidia** broadly ellipsoidal to subglobose or obovoid, 3.0–5.3 × 2.4–4.3 μm, appearing smooth but slightly rugose in SEM observations, pale brownish.

12. *Trichoderma* anamorph of *Hypocrea gelatinosa* (Tode: Fr.) Fr., Summa Veg. Scand. p. 383. 1849. (Fig. 1.14).

DESCRIPTION: Webster, Trans. Br. Mycol. Soc. 47:75–96. 1964.

Colonies growing relatively slowly (1.5–3.5 cm). **Conidiation** sparse and effuse, or forming numerous, small, irregular fascicles up to 2 mm diameter, slowly turning dull-green to blackish-green. Reverse colourless to yellow or orange. Odour faint, yeast-like. **Conidiophores** sparingly and simply branched; primary branches nearly uniform in length, usually arising in pairs and at regular intervals, apices of conidiophores and primary branches usually with a *Gliocladium*-like head comprised of 2–4 branches (1 or 2 cells in length) bearing verticils of 3–5 appressed phialides, or combinations of phialides and secondary branches. **Phialides** mostly lageniform to ampulliform, 5.5–12.0 × 2.7–3.3 μm, or terminal phialides subulate and up to 13 × 2–3 μm. **Conidia** obovoid to broadly ellipsoidal or subglobose, mostly 2.5–4.5 × 2.0–3.1 μm, smooth-walled, greenish; conidia from adjacent phialides often coalescing into large gloeoid masses.

13. *Trichoderma hamatum* (Bon.) Bain., Bull, trimest. Soc. mycol. Fr. 22:131. 1906. (Figs. 1.7, 8)

= *Verticillium hamatum* Bon., Handb. allg. Mykol. p. 97. 1851.

= *Pachybasium hamatum* (Bon.) Sacc., Rev. Mycol. Toulouse 7:160. 1885.

= *Phymatotrichum hamatum* (Bon.) Oud., Ned. kruidk. Archf 3(2):908. 1903.

Colonies growing moderately rapidly (3.5–6 cm). **Conidiation** in compact pustules, often distributed concentrically or aggregated near the colony margin; pustule surface appearing velvety due to numerous, flexuous, delicate, sterile conidiophore apices, bluish-green. Reverse colourless. Odour indistinct. **Conidiophores** typical of the section, the upper part sinuous to undulate or hamate, branching irregularly, sterile for about 100 μm to the apex. **Phialides** subglobose to ellipsoidal or ampulliform, 3.3–5.6 × 2.8–3.5 μm, mostly arising in crowded whorls of 3–6. **Conidia** oblong to ellipsoidal, 3.0–4.5 × 2.1–2.8 μm, smooth- and relatively thin-walled, dilute green.

Until 1991 many taxa were united in this species aggregate, but Bissett (1991b) defined the taxon more narrowly and designated a neotype for it.

14. *Trichoderma inhamatum* Veerkamp & W.Gams, Caldasia 13:710. 1983. (Fig. 1.4).

Colonies growing moderately rapidly (3.5–4.5 cm diam. in 6 days at about 20°C, up to 9 cm diam. in 3 days at 24–30°C). **Conidiation** finely floccose, partly in compact pustules, dark green to dark herbage-green, reverse colourless; pustule surface not appearing velvety and sterile conidiophore apices absent. Odour indistinct but typical of the genus. **Conidiophores** densely pyramidally branched, typical of the section but lacking sterile apices, 3.5–4 μm wide, subterminal branches 2.0–2.5 μm. **Phialides** lageniform, 4–5 × 2.3–3.0(–3.5) μm, mostly arising in crowded whorls of 3–6. **Conidia** globose to slightly ovoid, 2.3–3.0 × 2.0–2.6 μm, smooth- and relatively thin-walled, green. Temperature optimum 24–30°C, maximum 36°C.

Bissett (1991b) synonymized *T. inhamatum* with *T. harzianum*. The species was shown to be slightly distinct on molecular grounds (Gams and Meyer, 1998).

15. *Trichoderma longipile* Bissett, Can. J. Bot. 69:2395. 1991.

Colonies growing rapidly (4.5–6 cm). **Conidiation** in compact pustules, grey-green to olive-grey in age. Reverse colourless. Odour indistinct or faintly acrid. **Conidiophores** sparingly branched, primary branches relatively short, arising singly, alternately or in pairs, and sparingly rebranched; upper part of conidiophore main axis with a very long, nonfertile part, which is often highly ramified and anastomosing to within 100 μm of the apex; the apical part is unbranched and flexuous to strongly undulate. **Phialides** ampulliform to nearly subglobose, mostly 4.2– 6.0 × 3.0–4.5 μm, arising in whorls of 2–5 (usually in uncrowded whorls of 3), rarely solitary; terminal phialides more elongate and more or less lageniform. **Conidia** short-cylindrical to more or less oblong, sometimes slightly constricted at the middle, mostly 3.9–5.7 × 2.0–3.0 μm; smooth-walled, pale greyish.

16. *Trichoderma minutisporum* Bissett, Can. J. Bot. 69:2396. 1991. (Fig. 1.9).

Colonies growing rapidly (5–6 cm). **Conidiation** in compact, cushion-shaped pustules; appearing granular or mealy due to abundant conidiation, quickly turning bright green. Reverse colourless, or yellowish to drab or pale-vinaceous in patches. Odour indistinct. **Conidiophores** highly branched; primary branches short near the apex and increasing in length toward the base of the conidiophore, arising at regular intervals in whorls of 2–4; rebranched regularly to form a highly ramified, pyramidal structure; the upper part of the conidiophore is straight or slightly flexuous, fertile, and regularly branched to the apex. **Phialides** more or less ellipsoidal, 2.8– 4.3(–5.0) × 2.7–3.1(–4.4) μm, mostly arising in crowded whorls of 3–6. **Conidia** broadly ellipsoidal, mostly 2.5–3.3 × 1.8–2.3 μm; smooth- and thin-walled, pale green.

17. *Trichoderma oblongisporum* Bissett, Can. J. Bot. 69:2398. 1991. (Fig. 1.17).

Colonies growing rapidly (4.5–6 cm). **Conidiation** effuse, or forming compact pustules, appearing hairy due to numerous, usually unbranched, sterile conidiophore apices, dull green. Reverse colourless to pale yellowish. Odour faintly yeasty. **Conidiophores** sparingly branched, primary branches arising singly, alternately or in pairs, upper part of conidiophore main axis with a very long, straight or flexuous, nonfertile part. **Phialides** more or less ampulliform, 3.3–6.6 × 2.9–3.6 μm, uncrowded, usually arising in whorls of 2 or 3, occasionally solitary or in whorls of 4. **Conidia** ellipsoidal to oblong or short-cylindrical, 3.5–5.0 × 1.7–2.8 μm, smooth-and thin-walled, subhyaline to pale greenish.

18. *Trichoderma piluliferum* Webster & Rifai, in Rifai, Mycol. Pap. 116:16. 1969.

TELEOMORPH: *Hypocrea pilulifera* Webster & Rifai, Trans. Br. Mycol. Soc. 51: 511. 1968. (Fig. 1.15).

DESCRIPTIONS: Webster and Rifai (1968), Rifai (1969).

Colonies growing slowly (1.5–3 cm). Conidiation mostly in compact tufts, white. Reverse colourless to dull yellow. Odour indistinct. **Conidiophores** highly branched, branches usually 2- or 3-verticillate, arising at regular intervals, increasing in length toward the base of the conidiophore; upper part of conidiophore main axis straight to flexuous, regularly branched and fertile to the apex. **Phialides** usually arising in whorls of 2–5 at the apex of conidiophore branches, mostly ampulliform to nearly pyriform, 4.5–6.5 × 2.8–3.5 μm, or terminal phialides up to 10 μm long. **Conidia** globose to subglobose, 2.5–3.5 μm diam., smooth-walled, colourless.

19. *Trichoderma polysporum* (Link: Fr.) Rifai, Mycol. Pap. 116:18. 1969.

= *Sporotrichum polysporum* Link, Mag. Ges. naturf. Freunde, Berl. 7:34. 1815: Fries, Syst. mycol. 3:424. 1832. (Figs. 1.10–1.11).

TELEOMORPH: *Hypocrea pachybasioides* Doi, Bull. natn. Sci. Mus. Tokyo 15:685. 1972.

Colonies growing moderately rapidly (3–5 cm). **Conidiation** developing slowly (usually after 7–14 days), mostly in compact, cushion-shaped pustules, pustule surface appearing more or less velvety due to numerous, flexuous, sterile conidiophore apices; white to cream-coloured. Reverse colourless to dull yellowish. Odour indistinct or slightly mouldy. **Conidiophores** highly branched, primary branches mostly short, usually paired, occasionally in verticils of three, secondary branches in whorls of 2–4 (usually 3), upper part of conidiophore main axis persistently sterile, coarse, spiral-shaped; conspicuously tuberculate in most isolates especially toward the apex; occasional isolates with the conidiophore apex smooth-walled and with a solitary, terminal phialide, or a few simple branches terminated by phialides or whorls of phialides. **Phialides** usually arising in whorls of 2–5 at the apex of the terminal branches, less often laterally on the conidiophore and its branches, occasionally in crowded clusters of up to 7 on inflated terminal cells, mostly ampulliform 3.5–7 × 2.7–3.6 μm. **Conidia** ellipsoid, 2.3–3.6 × 1.4–2.2 μm, smooth-walled, colourless.

20. *Trichoderma pubescens* Bissett, Can. J. Bot. 69:2405. 1991.

Colonies growing moderately rapidly (3.5–6 cm). **Conidiation** in numerous, compact pustules, occasionally arranged concentrically, surface appearing downy due to numerous, thin, branched sterile conidiophore apices; bright-green to dull yellow-green. Reverse uncoloured or pale yellowish. Odour indistinct. **Conidiophores** typical of the section, upper part of conidiophore main axis persistently nonfertile, often branched, narrow, flexuous, or undulate to loosely coiled. **Phialides** ampulliform to lageniform, mostly 4.0–6.0 × 2.2–3.5 μm, 2–4-verticillate. **Conidia** ellipsoidal, 3.1–4.7 × 2.0–2.9 μm, dilute-green, wall smooth and relatively thin.

21. *Trichoderma* anamorph of *Hypocrea semiorbis* (Berk.) Berk., in Hooker, Fl. Tasm. 3(2):278. 1860 (Fig. 1.12).

Colonies growing slowly or sparsely (1.5–5 cm). **Conidiation** lacking or effuse on most media, on malt agar producing small, compact pustules, glaucous, or greyish in age. Reverse colourless to yellowish. Odour faintly aromatic, like pine pitch. **Conidiophores** relatively coarse and inflexuous, main axis regularly branched

over the lower (fertile) part with branches arising singly or more often in pairs or groups of three; branches short, usually 1–2-celled and rarely rebranched more than once; upper part of conidiophore nonfertile for 100–200 μm, relatively straight. **Phialides** broadly ampulliform, occasionally arising singly but usually 2–5-verticillate, mostly 4.7–8.0 × 3.0–4.1 μm. **Conidia** ellipsoidal to short-cylindrical, 3.0–4.6 × 1.8–2.7 μm, appearing smooth- and thin-walled, subhyaline to greyish.

22. *Trichoderma spirale* Bissett, Can. J. Bot. 69:2408. 1991.

Colonies growing rapidly (4–7 cm). **Conidiation** in compact, cushion-shaped pustules, blue-green or olive-grey in age. Reverse in some isolates colourless, most isolates developing dull-yellowish to reddish-brown shades. Odour indistinct or mouldy. **Conidiophores** comparatively little branched, branches relatively short throughout, mostly arising singly, alternately or paired, not extensively rebranched; apex of conidiophore stout and coiled over the last 30–100 μm. **Phialides** broadly ampulliform to nearly subglobose, mostly 3.3–5.2 × 2.8–4.0 μm, borne in whorls of 2–6 (mostly 3–4), usually not crowded. **Conidia** ellipsoid to more or less oblong, 3.0–4.4 × 1.8–2.7 μm, appearing smooth, at high magnifications (SEM) nearly smooth to minutely rugose, green.

The conidiophore apex in *T. spirale* has conspicuous rugose roughenings that disappear in liquid microscope mounts. In transmission electron microscope observations these tubercules appear empty and enclosed by diffuse, electron-dense material. They are not bounded by a membrane as they are in *T. polysporum* and *T. croceum*.

23. *Trichoderma strictipile* Bissett, Can. J. Bot. 69:2410. 1991.

Colonies growing moderately rapidly (3.5–6 cm). **Conidiation** in numerous, small, compact pustules, surface appearing hairy due to the presence of very long, relatively straight conidiophore apices, dull green. Reverse colourless. Odour indistinct. **Conidiophores** straight, branching dendritically, branches increasing in length toward the base of the conidiophore, usually rebranched several times, upper part of conidiophore main axis nonfertile for about 100 μm, straight or only slightly flexuous. **Phialides** mostly ampulliform, 3.4–7.5 × 2.6–4.0 μm, mostly arising in crowded whorls of 3–6. **Conidia** broadly ellipsoid, 2.8–4.0 × 2.2–3.0 μm, smooth- and thin-walled, dilute green.

24. *Trichoderma strigosum* Bissett, Can. J. Bot. 69:2411. 1991.

Colonies growing moderately rapidly (4–6 cm). **Conidiatio**n in compact pustules, surface appearing prickly or spiny at low magnifications due to the presence of conspicuous, stiff, javelin-like, sterile conidiophore apices; dull bluish-green. Reverse colourless to white or dull yellowish in age. Odour in some isolates faintly fragrant, like coconut, in other isolates faintly mouldy. **Conidiophores** straight and stout, highly branched with primary branches short, usually arising in whorls of 2 or 3 at acute angles and reflexed toward the apex of the conidiophore, usually highly rebranched; the conidiophore apices straight, stiff and javelin-like, rarely somewhat undulate, the apex usually bearing a solitary, unmodified, subulate conidiogenous cell 8–15 μm long and 1–2 μm wide. **Phialides** lageniform to ampulliform, 3.8–7.0 × 2.1–3.4 μm, usually arising in whorls of 2–4, occasionally arising singly. **Conidia** ellipsoidal to short-cylindrical, mostly 3.0–4.8 × 1.8–2.5 μm, green, smooth- and thin-walled.

25. *Trichoderma tomentosum* Bissett, Can. J. Bot. 69:2412. 1991.

Colonies growing moderately rapidly (4–6 cm). **Conidiation** in compact pustules; surface of pustules with a distinctly hairy appearance due to presence of numerous, spiral, sterile conidiophore apical elongations, grey-green. Reverse colourless or pale dull yellowish. Odour indistinct. **Conidiophores** coarse, highly branched; primary branches short, 2–4 cells long, arising in verticils of 2–4, final branches mostly 1-celled, swollen or barrel-shaped; the main axis with a conspicuous, sterile upper part which is strongly coiled or circinate near the apex. **Phialides** ampulliform to subglobose, mostly 3.5–5 × 2.5–3.5 μm, arising in whorls of 3(–5). **Conidia** broadly ellipsoid, mostly 2.5–3.4 × 1.9–2.3 μm, wall smooth and relatively thin, green.

26. *Trichoderma virens* (J.Miller, Giddens & Foster) von Arx, Beih. Nova Hedwigia 87:288. 1987. (Fig. 1.16).

= *Gliocladium virens* J.Miller, Giddens & Foster, Mycologia 49:792. 1957.

DESCRIPTION: Webster and Lomas (1964).

Colonies growing rapidly (6–7 cm). **Conidiation** mostly predominantly effuse, covering the entire surface of the plate, or forming spreading, flat pustules concentrated near the margin of the plate or arranged concentrically; quickly turning dark bluish-green. Reverse colourless, or slowly developing dull yellowish to amber shades. Odour indistinct. **Conidiophores** in areas of effuse conidiation arising as lateral branches from undifferentiated aerial mycelium, at the base frequently sterile and unbranched for about half the length, toward the apex branching irregularly with each branch terminated by a cluster of 3–6 closely appressed phialides; macronematous conidiophores branching irregularly, the upper part fertile to the apex and the apex frequently bearing a terminal whorl of appressed branches and phialides; primary branches usually arising singly or in opposite pairs immediately beneath septa, the entire branching system irregular and uncrowded. **Phialides** from complex conidiophores lageniform to ampulliform, mostly 4.5–10(–13) × 2.8–5.5 μm, mostly arising in closely appressed verticils of 2–5 on terminal branches, occasionally solitary or in pairs laterally on the conidiophore and branches; phialides from effuse areas of conidiation lageniform to subulate, up to 20 μm long × 2.5–3 μm. **Conidia** broadly ellipsoidal to obovoid, mostly 3.5–6.0 × 2.8–4.1 μm, smooth-walled (minutely warted at high magnifications—SEM, Meyer and Plaskowitz, 1989), dark green, conidia from adjacent phialides often coalescing into large gloeoid masses.

Because of the close affinity to the anamorph of *Hypocrea gelatinosa* (Tode: Fr.) Fr., classification of this species in *Trichoderma* is certainly preferable to that in *Gliocladium,* although the converging phialides are reminiscent of the latter genus (Webster and Lomas, 1964). This species is not to be confused with the *Gliocladium viride* Matr. and the similar anamorph of *Hypocrea lutea* (Tode) Petch, which have very pronounced erect conidiophore stipes with multiple verticils of densely penicillate branches, metulae and phialides.

Trichoderma* section *Longibrachiatum Bissett, Can. J. Bot. 62:925. 1984.

Colonies growing rapidly (4.5–7.5 cm), reverse in fresh isolates conspicuously yellowish-green. **Conidiation** effuse or loosely tufted, green. **Conidiophores** sparingly branched, primary branches long, secondary branches usually short and rarely rebranched. **Phialides** irregularly disposed, mostly solitary, occasionally in verticils of 2 or 3, ampulliform to lageniform or cylindrical; intercalary aphanophialides often produced immediately beneath terminal phialides. **Conidia** green, smooth-walled, ellipsoid to obovoid.

Type species: *Trichoderma longibrachiatum* Rifai.

27. *Trichoderma citrinoviride* Bissett, Can. J. Bot. 62:926. 1984. (Fig. 1.20).

TELEOMORPH: *Hypocrea schweinitzii* (Fr.: Fr.) Sacc., Sylloge Fungorum 2:522. 1883 [= *Sphaeria schweinitzii* Fr.: Fr., Elenchus Fungorum 2:60. 1829], *fide* Kuhls *et al.* (1997).

Colonies growing rapidly (4.5–7.5 cm). **Conidiation** usually more or less concentric or concentrated near the margin, bright green or yellowish-green. Reverse yellowish-green in fresh isolates. **Conidiophores** typical of the section. **Phialides** 3-verticillate, paired, or solitary and alternate, or irregularly disposed especially toward the apex of the conidiophore, lageniform to ampulliform, 3.5–6.6 × 2.0–3.2 μm, terminal phialides often narrowly conoidal and up to 12 μm long. **Conidia** pale green, ellipsoidal, 2.2–3.7 × 1.5–2.1 μm.

28. *Trichoderma longibrachiatum* Rifai, Mycol. Pap. 116:42. 1969. (Fig. 1.18).

TELEOMORPH: *Hypocrea* aff. *schweinitzii* [*fide* Kuhls *et al.* (1997)].

Colonies growing rapidly (6–7 cm). **Conidiation** fasciculate initially, eventually coalescent forming crusts, dark green; reverse usually pale greenish-yellow. **Conidiophores** typical of the section. **Phialides** solitary, paired or 3-verticillate, lageniform, 5.3–11.6 × 2.0–3.2 μm, or terminal phialides conoidal and up to 14 μm long, intercalary phialides frequently produced below the terminal phialides. **Conidia** pale to medium green, obovoid to ellipsoidal, 3.4–6.6 × 2.3–3.5 μm.

29. *Trichoderma parceramosum* Bissett, Can. J. Bot. 69:2418. 1991. (Fig. 1.22).

= *T. atroviride* Bissett, Can. J. Bot. 62:930. 1984 [non P.Karsten, 1892].

TELEOMORPH: *Hypocrea* aff. *schweinitzii, fide* Kuhls *et al.* (1997).

Colonies growing rapidly (8–9 cm). **Conidiation** mostly in numerous evenly distributed, compact pustules up to 2 mm diam., eventually often coalescent, rapidly turning dark green; reverse developing bright greenish-yellow pigments. Odour indistinct. **Conidiophores** typical of the section. **Phialides** mostly solitary, 5.2–10.0 × 2.1–3.3 μm, only slightly or not constricted at the base, terminal phialides more elongate, up to 14 μm long. **Conidia** comparatively dark green, more or less ellipsoidal, mostly 4.3–6.0 × 2.7–3.7 μm, but often mixed with narrower and paler ones, 5.0–6.0 × 2.0–2.5 μm.

30. *Trichoderma pseudokoningii* Rifai, Mycol. Pap. 116:45. 1969. (Fig. 1.21).

TELEOMORPH: *Hypocrea* aff. *schweinitzii* [*fide* Kuhls *et al.* (1997)].

Colonies growing rapidly (6–7 cm). **Conidiation** effuse, azonate or fasciculate, light green. Reverse uncoloured. Odour indistinct. **Conidiophores** typical of the section. **Phialides** 2–5-verticillate, or solitary and alternate, more irregularly disposed toward the apex of the conidiophore, lageniform, 3.8–7.6 × 2.0–3.3 μm, terminal phialides narrowly conoidal and up to 13.5 μm long. **Conidia** pale green, short-cylindrical to ellipsoidal, 2.5–4.5 × 1.7–2.6 μm.

Because of its rather common verticillate phialides, this species seems to take an intermediate position between this section and *Trichoderma* sect. *Trichoderma.*

31. *Trichoderma reeseii* E.G.Simmons, in Bigelow and Simmons (eds), Abstr. 2nd Int. Mycol. Congr, vol. 2:618. 1977. (Fig. 1.19).

TELEOMORPH: *Hypocrea jecorina* Berk. & Br., J. Linn. Soc. 14:112 (Fungi of Ceylon, No. 989). 1875 [*fide* Kuhls *et al.* (1997)].

Colonies growing rapidly (5.5–7 cm). **Conidiation** tardy and scattered in minute tufts, pale yellow-green. **Conidiophores** typical of the section, rarely rebranched.

Phialides cylindrical, or slightly inflated, mostly 5.5–8 × 2.0–3.7 μm. **Conidia** pale green, ellipsoid, (3.0–)3.5–4.5 × 2.3–3.0 μm.

The genomic distinctness of this species from *T. longibrachiatum* was proven by Kuhls *et al.* (1996, 1997) based on analysis of rDNA internal transcribed spacer sequences. *T. reesei* with its ellipsoidal conidia was regarded as a clonally propagating derivative of *H. jecorina,* while isolates of *H. jecorina* have more elongate conidia, 5.5–7.5 × 2.8–4.0 μm.

32. *Trichoderma ghanense* Doi, Y.Abe & J.Sugiyama, Bull. Nat. Sci. Mus., Tokyo, Ser.B 13:3. 1987.

Colonies growing rapidly (9 cm and more), reverse yellow. **Conidiation** effuse or in pulvinate tufts, grey-green to dark green. **Conidiophores** irregularly branched, not extensively rebranched. **Phialides** variously lageniform to more or less ampulliform, often curved, 5–9 × 2.6–3.6 μm, irregularly disposed, usually singly or alternately, or occasionally paired. **Conidia** narrow oval to subcylindrical, dark green, 5.6 8.2 × 2.9–3.8 μm, most smooth-walled, but a portion with pustulate, repanded or sinuous extensions of the outer layer of the conidial wall.

Kuhls *et al.* (1997) suggest a synonymy of *T. ghanense* with *T. parceramosum* based on homologous rDNA ITS sequences, but the pronounced conidial ornamentation allows a morphological distinction between the two taxa.

33. *Trichoderma saturnisporum* Hammill, Mycologia 62:112. 1970. (Fig. 1.23).

DESCRIPTION: Doi *et al.,* Bull. Nat. Sci. Mus. Tokyo 13:1–9. 1987.

Colonies growing rapidly (7–9 cm), reverse uncoloured or with yellow pigment diffusing into the medium. **Conidiation** effuse or in pulvinate tufts, grey-green to dark green. **Conidiophores** irregularly branched, not extensively rebranched. **Phialides** variously lageniform to more or less ampulliform, often curved, 6–14 × 2.5–3 μm, irregularly disposed, usually singly or alternately, or occasionally paired. **Conidia** subcylindrical, dark green, 3.6–5.8 × 2.8–3.4 μm, variously winged, pustulate or rugose, with ornaments up to 1.3 μm high, some almost smooth.

Trichoderma* section *Hypocreanum Bissett, Can. J. Bot. 69:2367. 1991.

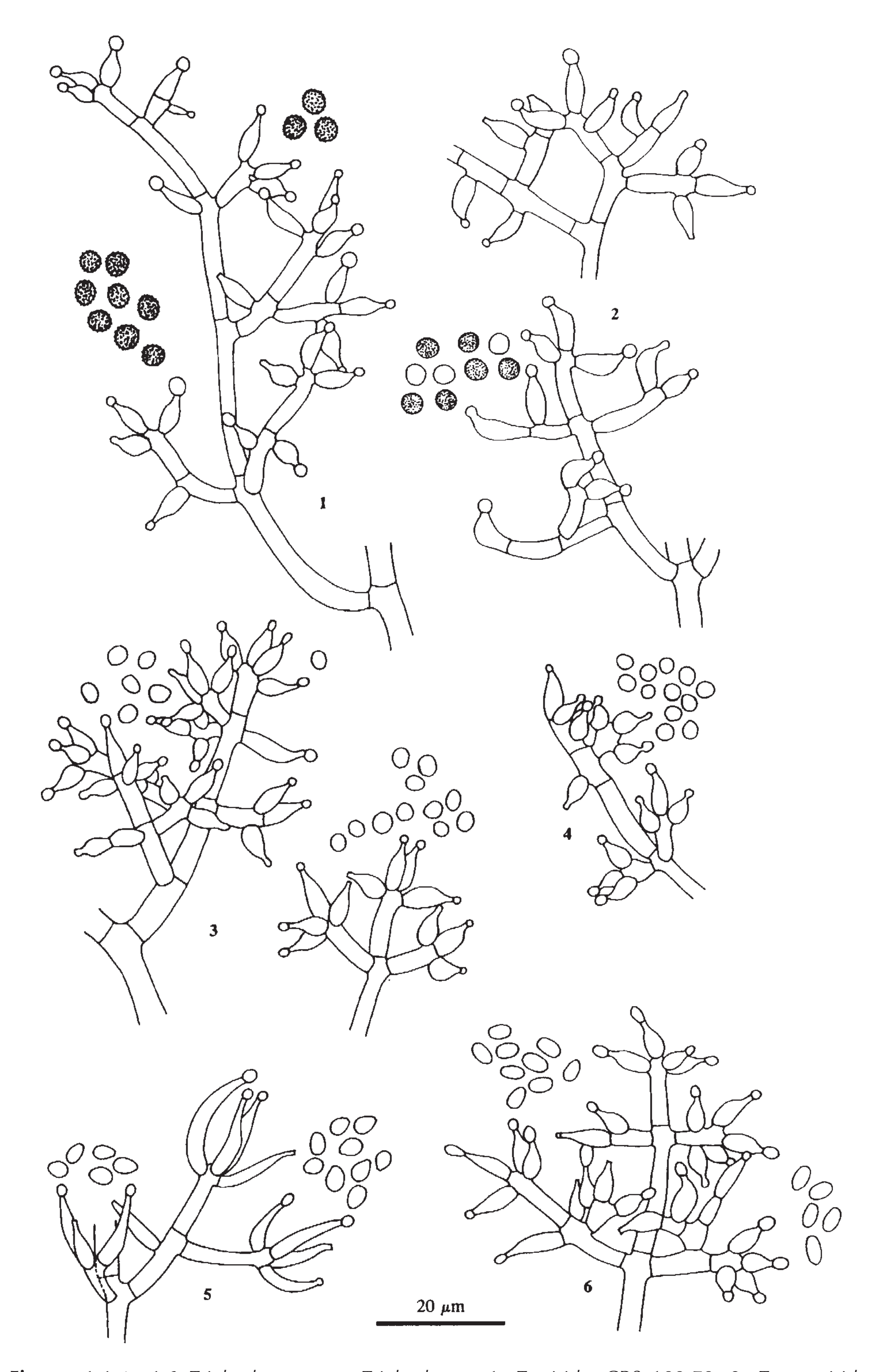
Colonies variable in growth rate, most species growing moderately (3–6 cm), reverse usually colourless to dull yellowish. **Conidiation** effuse, colourless or white. **Conidiophores** solitary, usually broad, irregularly and infrequently branched, occasionally unbranched except near the apex, not anastomosing. **Phialides** mostly borne in simple terminal verticils, occasionally solitary, cylindrical to lageniform. **Conidia** colourless, smooth-walled to slightly verrucose, more or less obovoid, or highly variable in shape and size. **Teleomorphs,** where known, in *Hypocrea.*

Type species: *Trichoderma lacteum* Bissett.

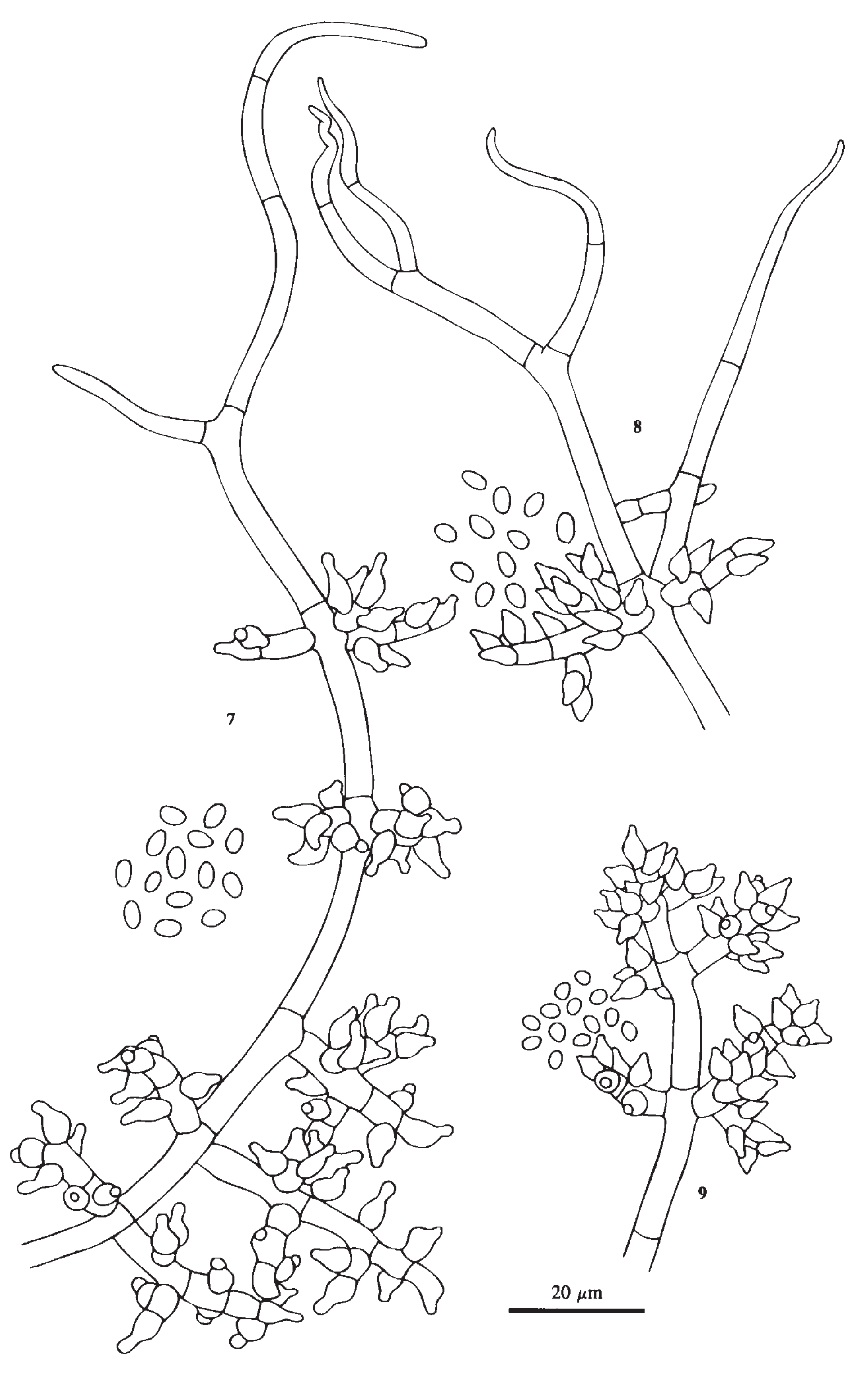
The *Hypocrea* anamorphs in *Trichoderma* section *Hypocreanum* are rarely observed independently of the teleomorph, and most have been discovered by culturing tissues or ascospores of species of *Hypocrea*. Many isolates eventually lose the capacity to produce conidia in culture. Species in section *Hypocreanum* share characters that may be mostly plesiomorphic (i.e. irregular conidiophore branching pattern, cylindrical phialides, effuse conidiation). The grouping of these forms in section *Hypocreanum* is probably not indicative of a natural relationship, although the majority of *Hypocrea* anamorphs referable to this section have teleomorphs in *Hypocrea* section *Homalocrea* (Sacc.) Doi (1972).

Acknowledgements

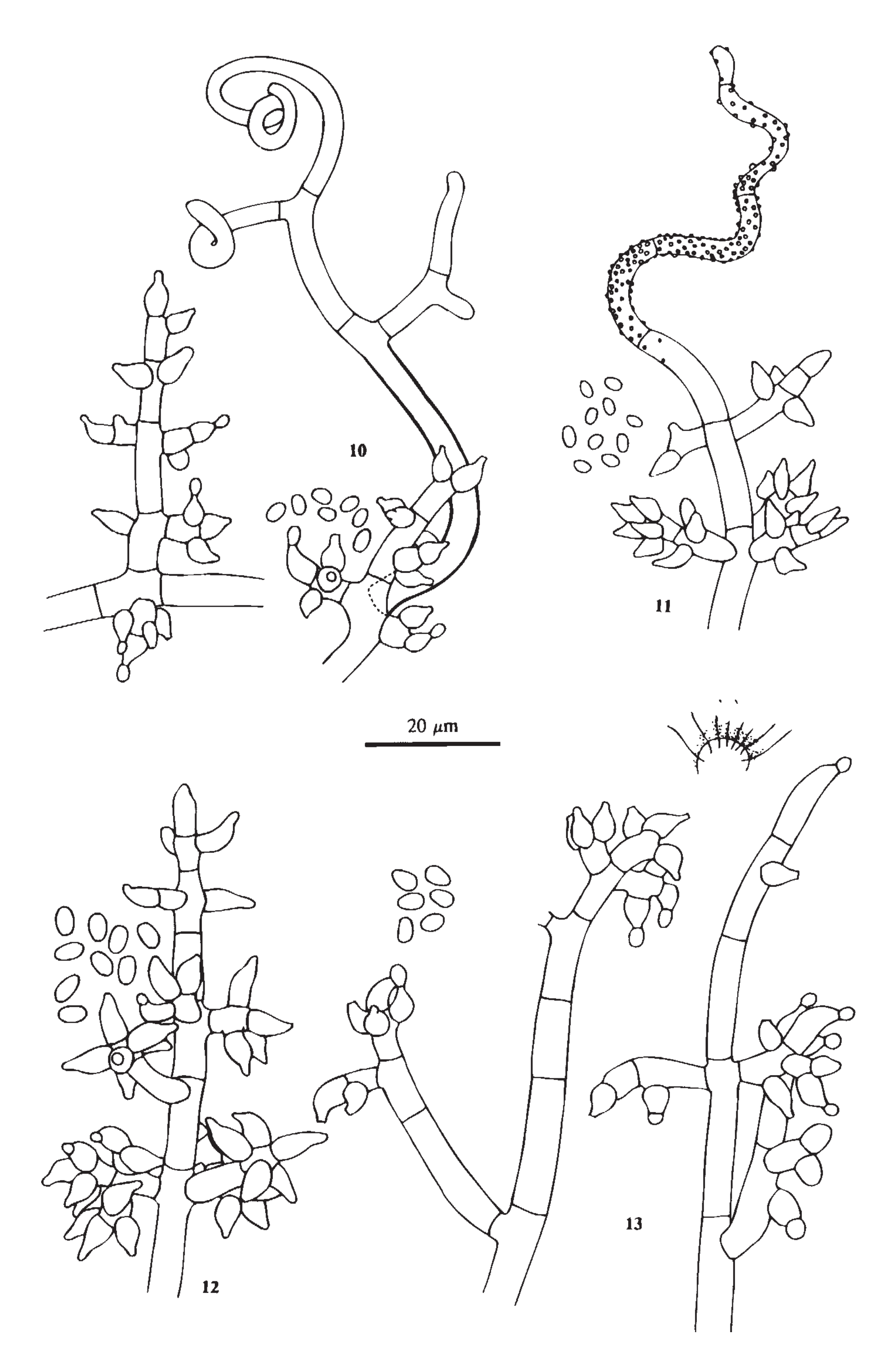
The authors are grateful to Drs G.J.Samuels and C.P.Kubicek for stimulating discussions and helpful comments. In addition, Dr Samuels provided numerous cultures for our study. Drs Katrin Kuhls and Kubicek generously provided access to their data and manuscripts in advance of their publication. We also thank Dr K.A. Seifert for helpful review of the manuscript, and Mrs C.C.van den Tweel-Vermeulen for inking the drawings.



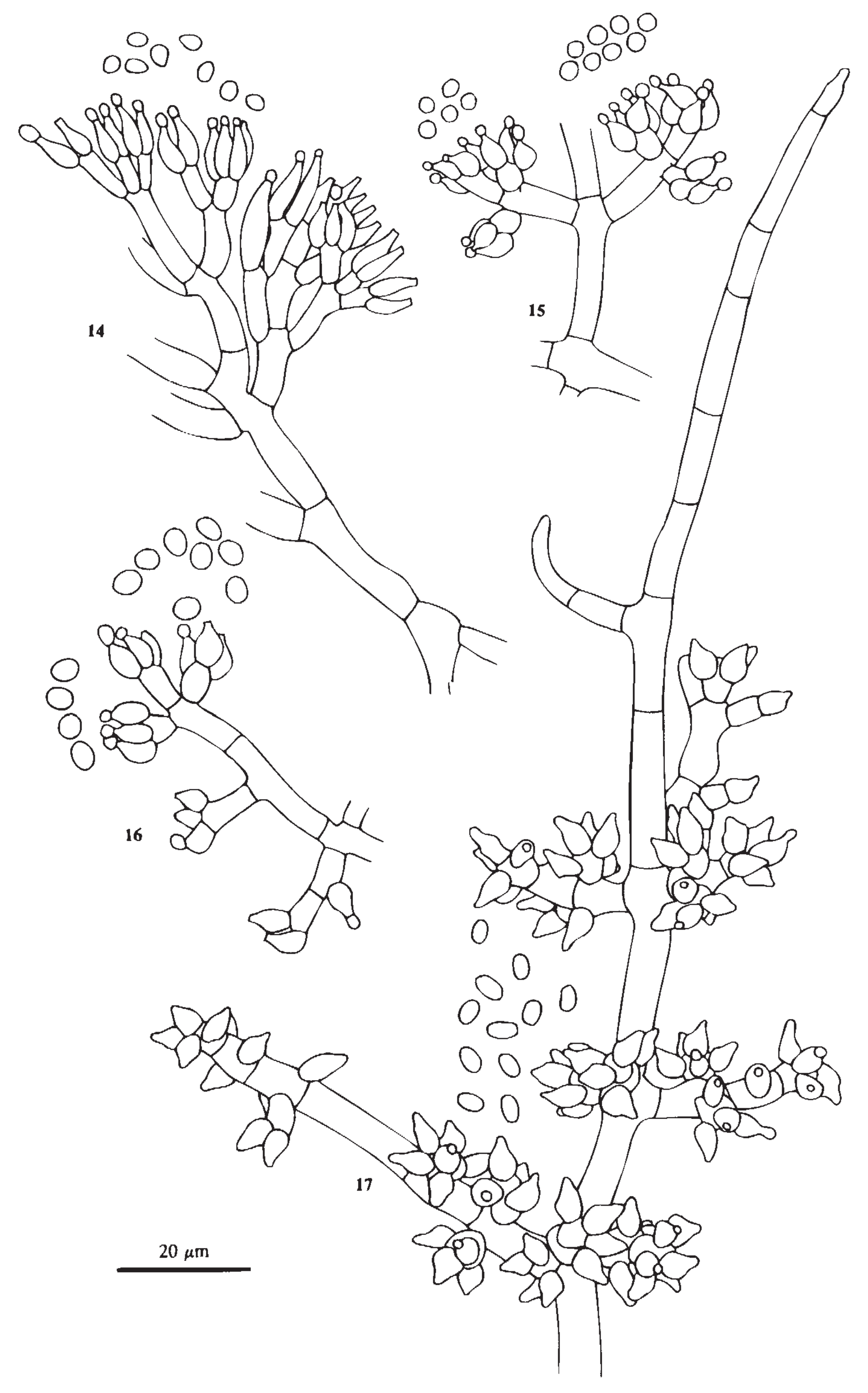
Figures 1.1 to 1.6 *Trichoderma* sect. *Trichoderma*. 1. *T. viride,* CBS 189.79. 2. *T. atroviride,* CBS 351.93. 3. *T. harzianum,* CBS 226.95. 4. *T. inhamatum,* CBS 274.72. 5. *T. aureoviride,* CBS 283.79. 6. *T. koningii,* CBS 457.96.



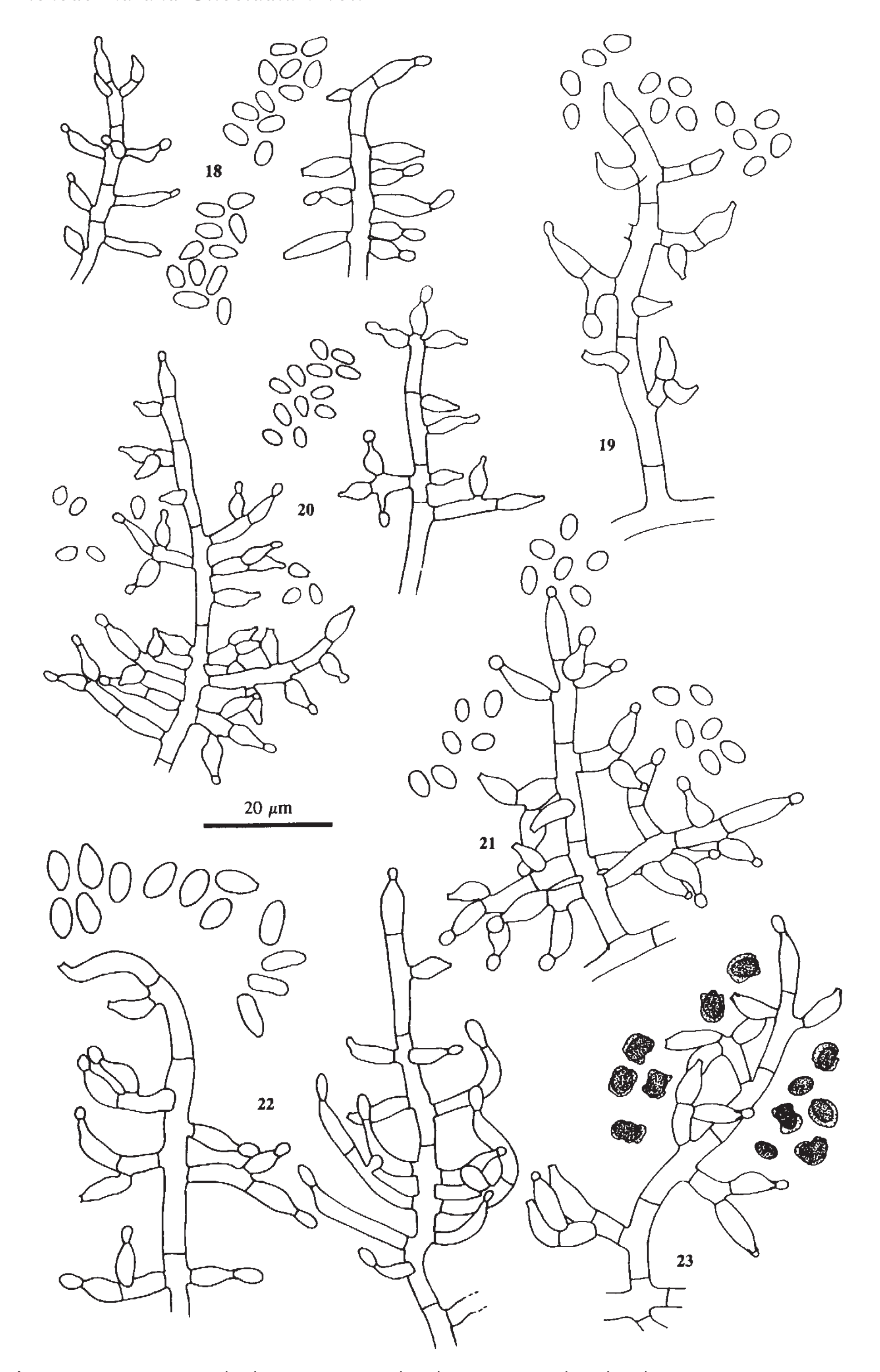
Figures 1.7–1.9 *Trichoderma* sect. *Pachybasium*. *7,* 8. *T. hamatum* DAOM 164925 and 175932 (redrawn from Bissett, 1991b). 9. *T. minutisporum,* DAOM 175931.



Figures 1.10–1.13 *Trichoderma* sect. *Pachybasium*. 10, 11. *T. polysporum,* CBS 147.69 and DAOM 167156, the latter (redrawn from Bissett, 1991b) showing a roughened sterile appendage. 12. *Hypocrea semiorbis,* DAOM 167636. 13. *T. fertile,* with a habit sketch of conidial pustule, CBS 339.93.



Figures 1.14 to 1.17 *Trichoderma* sect. *Pachybasium*. 14. *H. gelatinosa,* CBS 724.87. 15. *T. piluliferum,* CBS 224.84. 16. *T. virens,* CBS 497.84. 17. *T. oblongisporum*, DAOM 167085 (redrawn from Bissett, 1991b).



Figures 1.18 to 1.23 *Trichoderma* sect. *Longibrachiatum*. 18. *T. longibrachiatum,* CBS 816.68. 19. *T. reesei,* descendant of CBS 383.78. 20. *T. citrinoviride,* CBS 619.83 and 258.85. 21. *T. pseudokoningii,* CBS 408.91. 22. *T. parceramosum,* CBS 259.85 and 487.78. 23. *T. saturnisporum,* CBS 355.92.

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2

Molecular taxonomy of *Trichoderma* and *Gliocladium* and their teleomorphs

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"The very fact that some organisms in nature cannot easily be grouped into species is itself one of the most important pieces of evidence for the historical process we call evolution."
Charles Darwin

2.1 Introduction

Within the last 15 years, a new era of fungal systematics was initiated by the application of molecular techniques to answer questions of evolutionary patterns and processes. Classification of the genera *Trichoderma* and *Gliocladium* with teleomorphs within the *Hypocreales* has benefited tremendously from the application of molecular analyses.

Despite the importance of *Trichoderma* and *Gliocladium* in biocontrol and biotechnological applications (Volume 1, Chapter 1), individual species of both genera are poorly defined and classification of strains at the species level is difficult. Although the genus *Trichoderma* is easily recognized and has been extensively studied (Bissett, 1984, 1991a–c; Rifai, 1969), it has been difficult to reliably define species because of the apparent paucity of morphological characters. An account of the current morphologically-based key to species is given in Volume 1, Chapter 1.

Species of *Gliocladium* have been linked as anamorphs to at least six taxonomically rather distinct hypocrealean genera (Rehner and Samuels, 1994), and this has led to the conclusion that the genus is polyphyletic. Moreover, the morphologically defined borders between *Trichoderma* and *Gliocladium*—especially in terms of *Trichoderma (Gliocladium) virens*—are blurred (Rifai, 1969; von Arx, 1987). Therefore, in recent years molecular methods have been applied as an aid to resolving the taxonomy and systematics of *Trichoderma* and *Gliocladium*.

2.2 Macromolecular markers in *Trichoderma* and *Gliocladium* taxonomy—an overview

DNA data, and to a limited extent also protein data, reflect the genotype of the organism and may give a clearer picture of relationships than do morphological

characters. Molecular methods based on the characterization of proteins and/or nucleic acids and their polymorphisms provide an almost unlimited number of potential markers for taxonomic studies and reflect the phylogenetic relationship between organisms. Examples for the application of molecular methods in fungal systematics have been reviewed by Bruns *et al.* (1991) and Kohn (1992). Most of the methods have been applied in *Trichoderma* and *Gliocladium* taxonomy and are discussed in this chapter.

2.2.1 Protein markers (isozyme analysis)

A frequently used method for estimating genetic variation is isozyme electrophoresis. The first characterization of *Trichoderma* species by isozyme patterns was done by Zamir and Chet (1985). Twenty-three geographically diverse isolates of *T. harzianum* were grouped into five types according to the isozyme profiles. Results indicated that enzyme electrophoresis is useful for distinguishing *Trichoderma* at the intraspecies level. Stasz *et al.* (1989) used 16 enzyme loci that resolved 109 alleles in a study of 71 strains that were distributed between five morphological species (Rifai, 1969), namely *T. harzianum, T. hamatum, T. koningii, T. pseudokoningii* and *T. viride*. The authors found little correlation between the distribution of alleles and morphology. Samuels *et al.* (1994) and Leuchtmann *et al.* (1996) undertook a rather comprehensive investigation of *Trichoderma* section *Longibrachiatum* (Bissett, 1984) using isozyme characters. Samuels *et al.* (1994) used isozymes together with morphological and cultural characters to determine variation in the *Trichoderma* anamorph of *Hypocrea schweinitzii* and to assess its relationship to *T. longibrachiatum* Rifai, *T. reesei* Simmons and *T. pseudokoningii* Rifai. These studies were continued by Leuchtmann *et al.* (1996) who investigated 78 representatives of *Trichoderma* sect. *Longibrachiatum, H. schweinitzii* and *H. jecorina* Berk. & Broome. The results have shown the existence of biochemically defined groups that broadly confirmed the *Trichoderma* taxonomy of Bissett (1984).

2.2.2 DNA markers

Restriction fragment length polymorphisms and DNA fingerprinting

Polymorphisms at the DNA level can be studied by several means, the most common of which is the analysis of restriction fragment length polymorphisms (RFLPs). DNA is cut by enzymes (restriction endonucleases) that recognize specific DNA sequence motifs. Electrophoretic separation of the DNA fragments and hybridization to single or multilocus probes (the latter = DNA fingerprinting) reveals more or less complex polymorphic DNA patterns suited for taxonomic studies (Weising *et al.,* 1995). Mitochondrial DNA (mtDNA) and ribosomal DNA (rDNA) have been used extensively for evolutionary studies in fungi (reviewed in Bruns *et al.,* 1991). The high copy number of the sequences and their contents of conserved and variable parts are the reasons that both DNA types have been so popular.

Meyer (1991) used mitochondrial DNAs and plasmids as taxonomic characteristics for *T. viride.* He investigated 12 strains of the *T. viride* aggregate species and results of the molecular analyses were discussed in comparison to conidial ornamentation

(Meyer and Plaskowitz, 1989). Morawetz *et al.* (1992) and Kubicek *et al.* (1996) produced RFLP patterns of a DNA fragment representing the cellobiohydrolase I-encoding gene *cbh1* from different *Trichoderma* species that were species-specific. RFLPs of mtDNA and rDNA were used by Muthumeenakshi *et al.* (1994) to assess intraspecific variation between 81 *Trichoderma harzianum* isolates colonizing mushroom compost in the British Isles. Meyer *et al.* (1992) used multilocus probes in DNA fingerprinting experiments. They hybridized the synthetic oligonucleotides $(CT)_8$, $(GTG)_5$, $(GACA)_4$ and DNA of the wildtype bacteriophage M13 to restriction-digested genomic DNA of nine species of *Trichoderma*. These studies illustrated the need to reclassify some species of *Trichoderma*.

Random amplified polymorphic DNA- and PCR-based fingerprinting

The polymerase chain reaction (PCR) technique has created new ways of revealing DNA polymorphisms among closely related genotypes with high sensitivity via a fast and easy-to-perform protocol. PCR-based fingerprinting involves the amplification of unknown DNA fragments using arbitrary GC-rich primers, known as RAPD analysis (Williams *et al.,* 1990) and AP (artificially primed)-PCR (Welsh and McClelland, 1990) or microsatellite-complementary oligonucleotides: $(GACA)_4$, $(GTG)_5$ and M13 core sequence (Lieckfeldt *et al.,* 1993; Meyer *et al.,* 1992). PCR-based fingerprinting is widely applied for the characterization of *Trichoderma* strains and species for various purposes.

Schlick *et al.* (1994) and Kuhls *et al*. (1995) used PCR fingerprinting for *Trichoderma* strain identification and detection of culture impurities. The characterization of potential biocontrol strains of *Trichoderma* by PCR fingerprinting was the aim of Arisan-Atac *et al.* (1995), Fujimori and Okuda (1994), Turoczi *et al.* (1996) and Zimand *et al.* (1994). When analyzing soil isolates morphologically assigned to *T. harzianum, T. hamatum, T. viride* or *T. koningii,* all authors found two or more genetically distinct groups within each of these species. The RAPD technique was used by Muthumeenakshi *et al.* (1994) to investigate *Trichoderma harzianum* strains that are antagonistic to the commercial production of mushrooms. Kuhls *et al.* (1996) combined PCR fingerprinting with rDNA sequence studies to prove the relation between a strictly asexual *Trichoderma* species and the ascomycete *Hypocrea jecorina.* Turner *et al.* (1996) used the RAPD technique in an extensive study of molecular taxonomy of *Trichoderma* that included 145 strains of *Trichoderma* spp., *H. jecorina* and *H. schweinitzii* in an effort to align unidentified isolates with named *Hypocrea* and *Trichoderma* species.

DNA sequencing

DNA sequences provide large data sets. Because the sequences reflect the structure of the DNA itself, these data are considered to be more phylogenetically informative than other types of data. Moreover, comparisons of these data using the cladistic method and parsimony is considered to give a phylogenetically more reliable result than an analysis that are phenotypically based. Depending on the taxonomic level of interest, a more or less conserved genome region is chosen for sequence analysis. The gene-spacer-gene arrangement of the multicopy rDNA containing universally conserved regions (18S rRNA gene, 5.8S rRNA gene, 28S rRNA gene) as well as highly variable parts (e.g., internal transcribed spacer—ITS, intergenic

spacer—IGS) is well suited for such studies. Almost all sequence studies in fungi have focused on the rRNA genes (Bruns *et al.,* 1991).

Nucleotide sequence determination of ITS-1 in the genetically diverse *Trichoderma harzianum* by Muthumeenakshi *et al.* (1994) revealed distinct ITS types corresponding to the *T. harzianum* groups from RFLP and RAPD analyses found by the same authors. Analysis of ITS-1 sequences of 128 strains of *Trichoderma* revealed 15 distinct ITS types with 34 unique sequences recognizing *T. harzianum* (3 taxa), *T. viride, T. virens, T. hamatum* (2 taxa), *T. aureoviride, T. citrinoviride, T. longibrachiatum, T. parceramosum, T. polysporum, T. piluliferum, T. saturnisporum* and *T. reesei* (Muthumeenakshi, 1996).

A revision of *Trichoderma* sect. *Longibrachiatum* including related teleomorphs based on the analysis of rDNA ITS sequences was carried out by Kuhls *et al.* (1997). Variation in the ITS-1 and ITS-2 regions of 99 strains allowed the recognition of the morphologically defined species *T. longibrachiatum, T. pseudokoningii, T. parceramosum, T. citrinoviride,* all members of sect. *Longibrachiatum,* as well as *Hypocrea jecorina.* In order to evaluate the data with respect to taxon boundaries, the authors included *ex-type* cultures of most *Trichoderma* species of sections *Pachybasium, Trichoderma* and *Saturnisporum* in their studies.

Studies on molecular taxonomy and phylogeny of *Gliocladium* on the basis of nuclear large subunit rDNA sequences were done by Rehner and Samuels (1994). The phylogenetic distribution of *Gliocladium* species within the *Hypocreales* was investigated and two principal monophyletic groups resolved with anamorphs were classified in *Gliocladium*.

Chromosomes and karyotyping

The development of pulse-field gel electrophoresis (PFGE) has allowed the electrophoretic karyotyping of organisms. The technique has already been applied extensively to the separation of fungal chromosomes and to the study of the structural organization of fungal chromosomal DNA (reviewed in Mills and McCluskey, 1990). The fact that a high frequency of chromosomal length polymorphisms can be observed even at inter- and intraspecies levels led to the application of the method for phylogenetic studies.

Mäntylä *et al.* (1992) analyzed the wild-type and mutant strains of *T. reesei* and found striking differences in the karyotype of the mutants compared to the initial parent. The chromosomal bands were identified using different cellulase genes and revealed chromosomal rearrangements due to mutagenesis. Herrera-Estrella *et al.* (1993) have determined the electrophoretic karyotype of *T. harzianum, T. viride* and *T. reesei.* Chromosome size and pattern, as well as results from gene assignment, are discussed with respect to the evolutionary relationship between the three species. A current karyotype analysis with 10 isolates of *T. harzianum* and *T. reesei* was undertaken by Gomez *et al.* (1997). The authors could classify the isolates into six clearly distinct electrophoretic karyotypes. RAPD analysis confirmed this classification. The reported similarity between *T. reesei* QM9414 with one group of the *T. harzianum* isolates is striking and probably the result of confusion of isolates. Fekete *et al.* (1996) have used PFGE for karyotyping of three *Trichoderma* species, *T. atroviride, T. hamatum* and *T. koningii,* with respect to the location of the 42 kDa chitinase gene known from *T. harzianum*. Results indicate a polymorphic chromosomal location of the highly conserved gene within the genus *Trichoderma*.

Of the spectrum of molecular methods used in taxonomic studies, rDNA sequence analysis combined with RAPD/fingerprinting and mtDNA analysis seems most suited, whereas karyotyping becomes interesting when considering adjacent genes.

2.3 The species concepts of *Trichoderma* and *Gliocladium*—comparison of molecular data to morphologically-based classification systems

2.3.1 Definition of species limits in Trichoderma

In 1989 Meyer and Plaskowitz wrote that morphological or genetical characters that can reliably define biological species in *Trichoderma* have not been identified. Even today the question of an objective measure of the variability of any *Trichoderma* spp. is not answered. The comprehensive molecular studies of Samuels *et al.* (1994), Muthumeenakshi *et al.* (1994), Leuchtmann *et al.* (1996), Kuhls *et al.* (1996, 1997) and Turner *et al.* (1996) demonstrate that the genetic variation at different taxonomic levels can be characterized and that the combination of different molecular techniques for the purpose of taxonomy of a genus could be used for the definition of taxon boundaries in *Trichoderma*.

In the following paragraphs all molecular data reviewed in Section 2.1 are discussed in comparison to the differing species concepts in *Trichoderma* proposed, respectively, by Rifai (1969) and by Bissett (1984, 1991a–c). Rifai divided the genus into nine species aggregates and Bissett created five sections, each including several species of *Trichoderma*.

Homogeneity/integrity of section Longibrachiatum *according to Bissett*

The species set Results from molecular analyses largely support Bissett's (1984) morphologically-based analysis recognizing *Trichoderma* sect. *Longibrachiatum*. According to Bissett (1984, 1991c) sect. *Longibrachiatum* includes four *Trichoderma* spp. (*T. longibrachiatum* Rifai with *T. reesei* as a synonym, *T. pseudokoningii* Rifai, *T. citrinoviride* Bissett, *T. parceramosum* Bissett) and the anamorph of *Hypocrea schweinitzii (Fr.) Sacc.* The rDNA sequence studies of Kuhls *et al.* (1996, 1997) showed that sect. *Longibrachiatum* forms a strictly monophyletic group within *Trichoderma*. For each species of the section distinguished by Bissett the authors found a species-specific IST sequence. These findings are confirmed by the data from isozyme analysis of Leuchtmann *et al.* (1996) revealing the existence of four defined groups in agreement with the four species *T. longibrachiatum, T. parceramosum, T. citrinoviride* and *T. pseudokoningii*. RFLPs of rDNA and mtDNA recognized *T. longibrachiatum, T. citrinoviride* and *T. parceramosum* into discrete taxa and the *ex-type* isolate of *T. pseudokoningii* formed a subgroup in *T. citrinoviride*. *Trichoderma longibrachiatum* strains were also further divided into two subgroups (Muthumeenakshi, 1996). The latter is confirmed by data of Kuhls *et al.* (1997) and Leuchtmann *et al.* (1996). Turner *et al.* (1996) also distinguished these species of sect. *Longibrachiatum* by specific RAPD patterns. The analysis of related *Hypocrea* species further supports the results (Kuhls *et al.,* 1997). An

interesting fact is the somewhat separated position of *T. parceramosum* within sect. *Longibrachiatum* due to sequence differences in the ITS-2 region of the rDNA (Kuhls *et al.*, 1997).

Figure 2.1 summarizes the relationship between species of sect. *Longibrachiatum* inferred from isozyme and DNA data. Some of the strains morphologically identified as *T. pseudokoningii* (exclusive of the *ex-type* strain) had molecular features

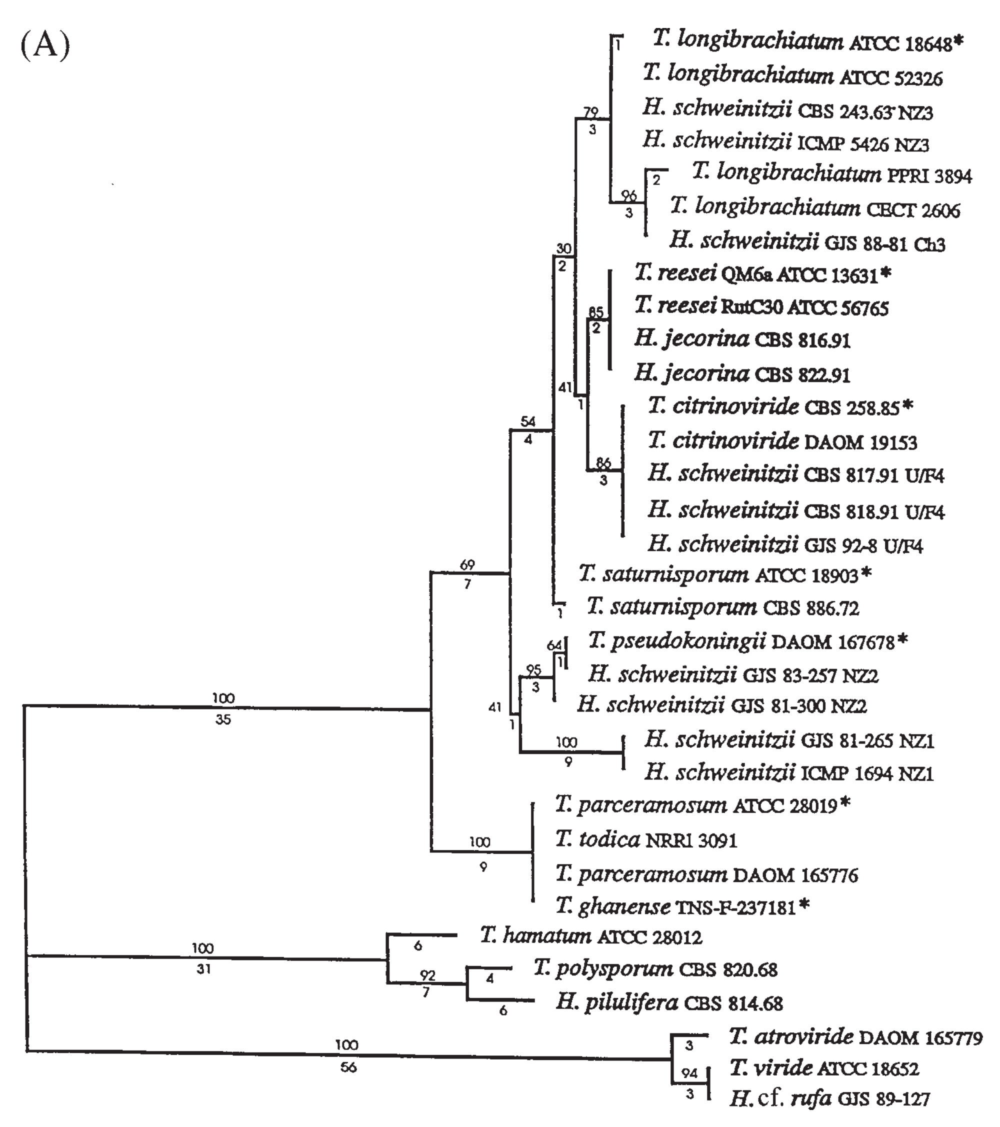


Figure 2.1 Phylogenetic trees derived from molecular data of *Trichoderma* spp. of sect. *Longibrachiatum* and of the teleomorphs *Hypocrea schweinitzii* and *H. jecorina*. (A) ITS-1-5.8S-ITS-2 sequences (from Kuhls *et al.*, 1997). The phylogram is the 50% majority rule consensus of the 9 most parsimonious trees generated by branch-and-bound analysis. Numbers above the branches are the bootstrap values after 500 replications, the values below the branches show the total nucleotide changes assigned to each branch. Asterisks indicate *ex-type* strains. (B) Isozyme data (reproduced with permission from Leuchtmann *et al.*, 1996). Dendrogram resulting from UPGMA analysis showing phenetic similarity in allozyme profiles. For abbreviations and strain numbers see Table 2.1.

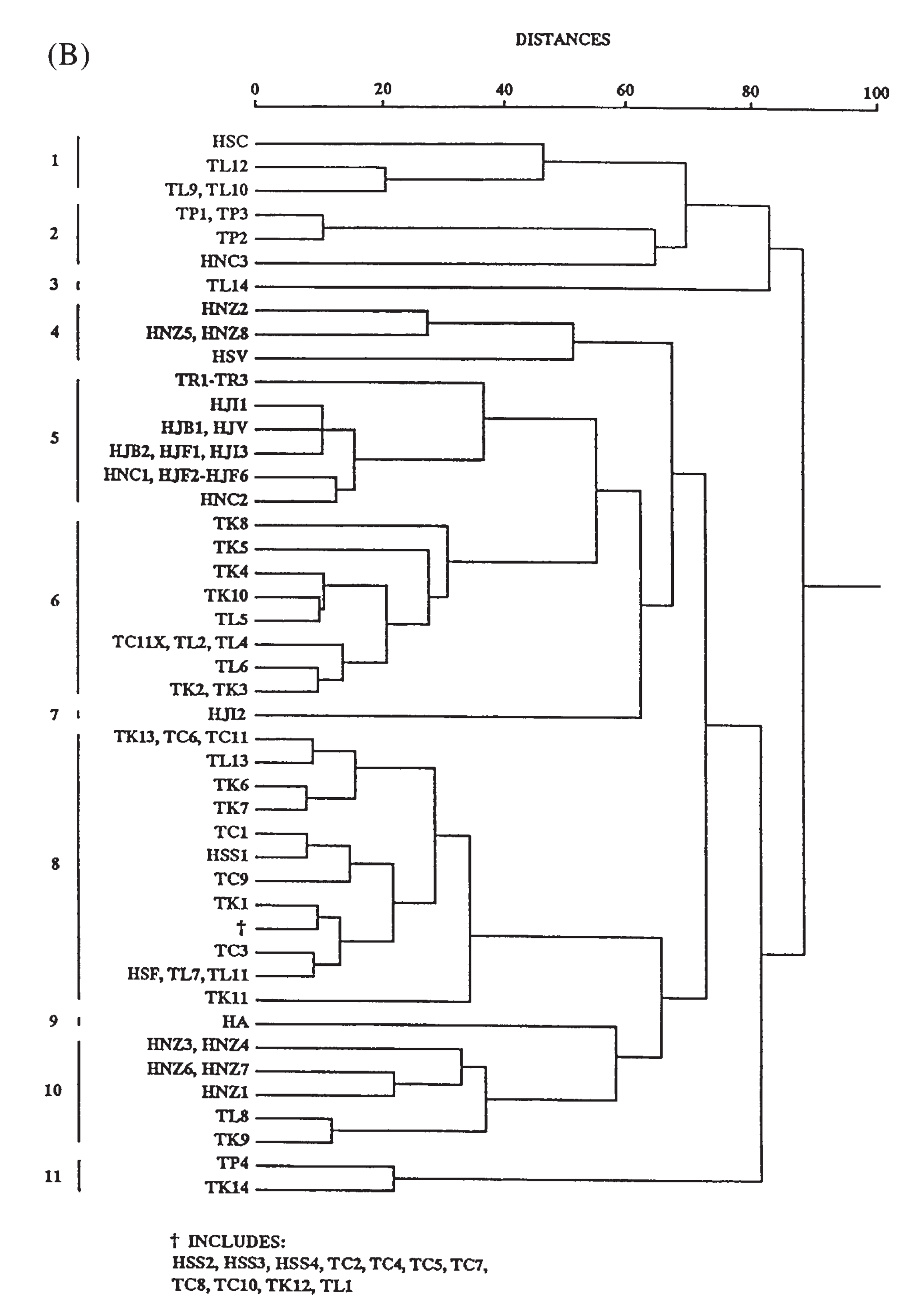


Figure 2.1 *(cont)*

Table 2.1 Strain numbers, species designation, geographic origin, and abbreviation code (as used in Fig. 2.1) (from Leuchtmann *et al.,* 1996).

Code	Strain	Species	Geographic origin	Code	Strain	Species	Geographic origin
HA	GJS 91-141	*H.?aurantia*	U.S.A.	TC9	DAOM 215099	*T. citrinoviride*	locality unknown
HJB1	GJS 84-473	*H. jecorina*	Brazil	TC10	EN 73	*T. citrinoviride*	U.S.A
HJB2	GJS 89-7	*H. jecorina*	Brazil	TC11X	DAOM 172792	*T. citrinoviride* (ex type)	Canada
HJF1	GJS 86-403	*H. jecorina*	French Guiana	TC11	CBS 258.85	*T. citrinoviride* (ex type)	Canada
HJF2	GJS 86-404	*H. jeocrina*	French Guiana	TK1	IMI 91793	*T. pseudokoningii*	U.K.
HJF3	GJS 86-408	*H. jecorina*	French Guiana	TK2	IMI 92027	*T. pseudokoningii*	U.K.
HJF4	GJS 88-6	*H. jecorina*	French Guiana	TK3	IMI 232088	*T. pseudokoningii*	U.K.
HJF5	AYR-2819	*H. jecorina*	French Guiana	TK4	IMI 287096	*T. pseudokoningii*	India
HJF6	AYR-2896	*H. jecorina*	French Guiana	TK5	IMI 287591	*T. pseudokoningii*	Nigeria
HJI1	GJS 85-229	*H. jecorina*	Indonesia	TK6	IMI 288111	*T. pseudokoningii*	Philippine Islands
HJI2	GJS 85-238	*H. jecorina*	Indonesia	TK7	IMI 288113	*T. pseudokoningii*	Philippine Islands
HJI3	GJS 85-249	*H. jecorina*	Indonesia	TK8	IMI 291014	*T. pseudokoningii*	Antarctica
HJV	CTR 72-94	*H. jecorina*	Venezuela	TK9	ATCC 64400	*T. pseudokoningii* (ex type)	Australia
HNC1	GJS 92-19	*H. jecorina*	New Caledonia	TK10	GJS 91-10	*T. pseudokoningii*	Brazil
HNC2	GJS 93-23	*H. jecorina*	New Caledonia	TK11	L. J. Mihuta 127	*T. pseudokoningii*	U.S.A.
HNC3	GJS 93-24	*H. jecorina*	New Caledonia	TK12	EN 34	*T. pseudokoningii*	U.S.A.
HNZ1	ICMP 5421	*H. schweinitzii*	New Zealand	TK13	EN 49	*T. pseudokoningii*	U.S.A.
HNZ2	ICMP 1694	*H. schweinitzii*	New Zealand	TK14	TSCH	*T. pseudokoningii*	Switzerland
HNZ3	ICMP 5426	*H. schweinitzii*	New Zealand	TL1	DAOM 139758	*T. longibrachiatum*	Canada
HNZ4	CBS 243.63	*H. schweinitzii*	New Zealand	TL2	DAOM 162162	*T. longibrachiatum*	Canada
HNZ5	GJS 81-265	*H. schweinitzii*	New Zealand	TL3	DAOM 166989	*T. longibrachiatum*	Canada
HNZ6	GJS 83-257	*H. schweinitzii*	New Zealand	TL4	DAOM 175937	*T. longibrachiatum*	Canada
HNZ7	CJS 81-300	*H. schweinitzii*	New Zealand	TL5	DAOM 167674	*T. longibrachiatum* (ex type)	U.S.A.
HNZ8	GJS 81-264	*H. schweinitzii*	New Zealand	TL6	DAOM 175956	*T. longibrachiatum*	Canada
HSC	GJS 88-81	*H. cf. jecorina*	P.R. China	TL7	MVHC 6587	*T. longibrachiatum*	Uruguay
HSF	GJS 92-8	*H. schweinitzii*	France	TL8	DAOM 210151	*T. longibrachiatum*	U.S.A.
HSS1	GJS 90-111	*H. schweinitzii*	U.S.A.	TL9	GJS 91-157	*T. longibrachiatum*	New Zealand
HSS2	CTR 79-290	*H. schweinitzii*	U.S.A.	TL10	GJS 91-158	*T. longibrachiatum*	Switzerland
HSS3	CTR 79-225	*H. schweinitzii*	U.S.A.	TL11	MVHC 6662	*T. longibrachiatum*	Uruguay
HSS4	GJS 93-1	*H. schweinitzii*	U.S.A.	TL12	PPRI 3894	*T. longibrachiatum*	South Africa
HSV	GJS 90-140	*H. schweinitzii*	Venezuela	TL13	EN 3	*T. longibrachiatum*	U.S.A.
TC1	DAOM 145647	*T. citrinoviride*	U.S.A.	TL14	EN 38	*T. longibrachiatum*	U.S.A.
TC2	DAOM 167676	*T. citrinoviride*	Canada	TP1	DAOM 165773	*T. parceramosum*	U.S.A.
TC3	GJS 91-167	*T. citrinoviride*	Venezuela	TP2	DAOM 165776	*T. parceramosum*	U.S.A.
TC4	DAOM 183926	*T. citrinoviride*	Canada	TP3	DAOM 190843	*T. parceramosum*	Canada
TC5	DAOM 175952	*T. citrinoviride*	Canada	TP4	DAOM 196932	*T. parceramosum*	Canada
TC6	DAOM 191523	*T. citrinoviride*	locality unknown	TR1	DAOM 167654	*T. reesei* (ex type), QM6a	Solomon Islands
TC7	DAOM 196431	*T. citrinoviride*	locality unknown	TR2	IMI 192654	*T. reesei*	Solomon Islands
TC8	DAOM 215084	*T. citrinoviride*	locality unknown	TR3	ATCC 56765	*T. reesei*	mutant RUT C30

characteristic of *T. longibrachiatum*. Similarly, some strains identified primarily as *T. longibrachiatum* show *T. citrinoviride*-like sequences (Kuhls *et al.,* 1997). This reflects the morphological similarity of all species placed in sect. *Longibrachiatum.*

T. reesei—*an additional species of sect.* Longibrachiatum *T. reesei* is known only from a single strain (QM6a) which served as the sole progenitor of many mutants that have been used for cellulase production. Initially *T. reesei* was distinguished from *T. viride* Pers.: Fr. and *T. longibrachiatum* on the basis of morphological and enzymatic characters (Simmons, 1977). Bissett (1984), considering only morphology, synonymized *T. reesei* under *T. longibrachiatum*. This synonymy was not supported by macromolecular data. All macromolecular data provide strong evidence that *T. reesei* and *T. longibrachiatum* are separate species, both belonging to sect. *Longibrachiatum*. RFLP analysis of *T. reesei* and *T. longibrachiatum* strains (Morawetz *et al.,* 1992), DNA fingerprinting (Meyer *et al.,* 1992), isozyme analysis (Leuchtmann *et al.,* 1996; Samuels *et al.,* 1994), rDNA and mtDNA RFLPs, RAPD and ITS-1 sequence analyses (Muthumeenakshi, 1996) and RAPD analyses, as well as ITS sequence data (Kuhls *et al.,* 1996), show that *T. reesei* is different from *T. longibrachiatum* with differences in the range of interspecies level. Moreover, it was shown by Samuels *et al*. (1994), Leuchtmann *et al.* (1996) and Kuhls *et al.* (1996) that *T. reesei* is apparently the anamorph of *H. jecorina*.

"T. todica" —*conspecific with* T. parceramosum *"T. todica"* is represented only by a single strain described in a US patent application (patent no. 3,323,966, 1967, United States Patent Office). The name is not published and has not been included in any *Trichoderma* classification scheme. Morphologically, *"T. todica"* is indistinguishable from *T. parceramosum*. Further evidence for identification of this strain as *T. parceramosum* is given by molecular features: similar PCR fingerprinting patterns (Kuhls *et al.,* 1995) and RFLP data (Muthumeenakshi, 1996) as well as identical ITS-1 and ITS-2 sequences of the rDNA of *T. todica* and *T. parceramosum* (Kuhls *et al.,* 1997).

Section Saturnisporum

Section *Saturnisporum* of the genus *Trichoderma* was introduced by Doi and coworkers (1987), based on striking conidial ornamentation of the two included species, *T. saturnisporum* and *T. ghanense*. Only a single strain of *T. ghanense* (TNS-F-237181) is known. The section was accepted by Bissett (1991b), although he mentioned the *Longibrachiatum*-like conidiophores and conidiophore branching system of *T. ghanense* and *T. saturnisporum*. According to the RFLP and RAPD analyses (Muthumeenakshi, 1996) and the sequence data of Kuhls *et al.* (1997), *T. saturnisporum* represents an additional species of sect. *Longibrachiatum*. Although *T. ghanense* is identical with *T. parceramosum* in sequence data and very similar in RAPD patterns, it can be distinguished from that species by the somewhat larger and more tuberculate conidia in the former. This is an example of morphology not reflecting the phylogenetic relationship. From molecular data it is clear that *T. ghanense* and *T. parceramosum* are at least extremely closely related if not conspecific. One can postulate that there is one strain of *T. parceramosum* that underwent mutation to give warted conidia, resulting in a strain described as *T. ghanense*. A similar mutation could have given

T. saturnisporum warted conidia. It might be expected to find a similar gene in closely related but taxonomically distinct fungi that would undergo mutation resulting in similar morphological patterns.

RAPD analyses of Turner *et al.* (1996) also indicate a close relationship of *T. saturnisporum* and *T. ghanense* to sect. *Longibrachiatum*.

Heterogeneity and overlapping of species of sections Trichoderma *and* Pachybasium

Morphological and molecular relationship between sections Trichoderma *and* Pachybasium Section *Trichoderma* Bissett accommodates *T. koningii, T. viride, T. atroviride* Karsten and *T. aureoviride*. The species vary most conspicuously in conidial shape and to a lesser degree in conidiophore branching (Bissett, 1991a). Section *Pachybasium* was created by Bissett (1991a) to accommodate morphologically similar forms that have been included in the *T. hamatum, T. polysporum, T. piluliferum* and *T. harzianum* species aggregates of Rifai (1969). The section is based on *Pachybasium* Sacc. (Saccardo, 1885) and was originally proposed to unify *T. hamatum* and *T. polysporum*.

Members of *Trichoderma* sections *Pachybasium* and *Trichoderma* are cosmopolitan and abundant soil inhabitants. Mainly due to their economic importance, many efforts have been made to characterize species of both sections by molecular approaches (see above). All data obtained so far show a high genetic variability at interspecies as well as intraspecies levels. Molecular analyses of species belonging to sections *Trichoderma* and *Pachybasium* had revealed that these two sections contain phylogenetically related species of one in the other (Kuhls *et al.,* 1997; Muthumeenakshi, 1996). *T. atroviride* Karsten, *T. koningii, T. viride* and morphologically similar *Hypocrea* anamorphs (Kuhls *et al.,* 1997) as well as part of *T. harzianum* (group 3 according to Muthumeenakshi *et al.,* 1994) are closely related to each other in ITS sequences with hardly any genetic distance to separate them. According to Muthumeenakshi (1996), *T. polysporum* and *T. piluliferum* are distinctly different and are genetically divergent from *T. hamatum,* which is closely related to the *T. viride* complex. The phylogenetic analysis of ITS-1 sequences placed the *T. viride* complex and *T. hamatum* in one clade, the *T. harzianum* complex in another and *T. polysporum* and *T. piluliferum* in a third clade as different as separate sections. Kuhls *et al.* (1997) found that strains of sect. *Pachybasium* were divided between two well-supported clades (1. *T. hamatum, T. polysporum, H. pilulifera;* 2. *T. harzianum, T. inhamatum*), showing sequence divergence in ITS-1 and ITS-2 sequences in the range of different sections. Morphology does not reflect the apparent genetic distance between the groups. Moreover, part of sect. *Pachybasium* morphologically characterized as *T. harzianum* and *T. hamatum* is genetically similar to sect. *Trichoderma*. A comprehensive reassessment of species relationships in *Trichoderma* sect. *Pachybasium* and including all the species derived from *T. hamatum* by Bissett (1991c) has been done by Kindermann *et al.,* 1998. Molecular markers clearly revealed that *H. aureoviridis* (anamorph = *T. aureoviride,* sect. *Trichoderma*) is very distantly related to the rest of the species of *Trichoderma* and its position along with the *T. viride* complex is not supported. The genetic diversity of the aggregate species included in sections *Pachybasium* and *Trichoderma* has been well demonstrated by many workers (Arisan-Atac *et al.,* 1995; Fujimori and Okuda, 1994; Gomez *et al.,* 1997; Muthumeenakshi *et al.,* 1994; Turoczi *et al.,* 1996; Zamir and Chet,

1985; Zimand *et al.,* 1994). The phylogenetic analyses of enzyme and DNA data did not result in the resolution of monophyletic groups corresponding to the morphologically-based species of sect. *Pachybasium* and *Trichoderma*. A reclassification of both sections according to molecular and morphological data and the redefinition of some of the species is in progress.

Heterogeneity of T. harzianum Morphotaxonomically, *T. harzianum* is the smooth-spored counterpart of *T. viride*. However, a close affinity of some strains of *T. harzianum* to *T. viride* was noted by Rifai (1969), who considered morphoplogical and cultural characters, and by Okuda *et al.* (1982) based on secondary metabolites in addition to morphology. Rifai (1969) also recognized the difficulties in distinguishing isolates of *T. aureoviride* from *T. harzianum.* Bissett (1991a) included the *T. harzianum* species aggregate in sect. *Pachybasium* and synonymized *T. inhamatum* with it. The nearly 1000 isolates of *T. harzianum* examined by Bissett in culture showed limited variation in conidiophore and conidial morphology and little apparent morphological affinity with other species in sect. *Pachybasium*. Bissett also noted that isolates with effuse conidiation could be mistaken for species in Rifai's *T. koningii* and *T. aureoviride* aggregates and that *T. atroviride* Karsten can show similarities in colony character to *T. harzianum* (Bissett, 1991c). Molecular investigation of strains belonging to these species demonstrated the misidentification of strains caused by ambiguous overlapping of morphological characters (Kuhls *et al.,* 1997; Muthumeenakshi, 1996).

T. harzianum is the most common species in the genus and for two reasons it is perhaps the most economically important species of *Trichoderma:* some strains are used in the biological control of plant disease-causing fungi, and other strains are severe antagonists in the commercial production of mushrooms. Molecular characterization of strains of *T. harzianum* has revealed considerable variation within this species aggregate (Fujimori and Okuda, 1994; Muthumeenakshi *et al.,* 1994) which is often related to differences in antibiotic or antagonistic activity (Fujimori and Okuda, 1994; Gomez *et al.,* 1997; Muthumeenakshi *et al.,* 1994; Turoczi *et al.,* 1996; Zamir and Chet, 1985; Zimand *et al.,* 1994). Unfortunately neither the type specimen of this species, nor any culture derived from the type specimen, can now be located. Thus the selection of a neotype was essential in order to establish a basis for this important species. Neotypification of *T. harzianum* was proposed by Gams and Meyer (1995), who selected the isolate CBS 226.95.

There are four discrete rDNA sequence groups, well supported by RFLP and RAPD markers within the *T. harzianum* complex (Muthumeenakshi and Mills, 1996). *T. harzianum* group 1 is found to be worldwide in distribution and comprises many reported biological control agents (Muthumeenakshi, 1996). According to Gams and Meyer (1995), the molecular pattern of the neotype isolate coincided with this group and also with Fujimori and Okuda (1994) type 2. Groups 2 and 4 of *T. harzianum* are aggressive mushroom colonizers and were found to be exclusive to the geographic location (Muthumeenakshi and Mills, 1996). These three groups of *T. harzianum* are phylogenetically closely related to each other, and the remaining group 3 is genetically similar to *T. koningii* and *T. viride* (Muthumeenakshi *et al.,* 1998). The connection of some isolates of the *T. harzianum* aggregate and sect. *Trichoderma* species was also described by Kuhls *et al.* (1997) from RFLP and sequence analyses of ITS-1 and -2. A close evolutionary relationship between *T. harzianum* and *T. viride* is suggested by similarity of electrophoretic

karyotype of both species (Herrera-Estrella *et al.,* 1993). From a two base sequence difference in ITS-1, Gams and Meyer (1998) conclude that *T. harzianum* group 1 and *T. inhamatum* are two separate species. On the other hand, the ITS sequence variation of the *ex-type* material of *T. inhamatum* was found to fall within the range of *T. harzianum* group 1 (Kuhls *et al.,* 1997), well-supported by rDNA RFLP grouping as well (Muthumeenakshi, 1996). The RFLP patterns of mitochondrial DNA of these two were highly similar, supporting Bissett's (1991b) view that they are not two different species. Molecular as well as morphological data clearly demand redefinition of the *T. harzianum* complex.

Heterogeneity of T. hamatum Rifai (1969) described the *T. hamatum* aggregate on the basis of the formation of a sterile, whip-like appendage on the conidiophore and in part on the formation of typically clustered and squat phialides. Primary conidiophores are typically formed in tufts, while secondary conidiophores may form apart from the tufts. Rifai referred to branching of some of the secondary conidiophores as being of the *T. koningii* type, although phialides of *T. koningii* are morphologically distinguished from phialides of the *T. hamatum* type. Okuda *et al.* (1982) suggested an association of *T. hamatum* with *T. viride* because some strains identified as *T. hamatum* in their study produced a coconut odour. Bissett (1991a) placed the *T. hamatum* aggregate of Rifai into sect. *Pachybasium* of his classification and derived 10 species from it.

Molecular data for *T. hamatum* strains are limited in comparison to *T. harzianum* or *T. viride*. Nevertheless, these data indicate heterogeneity of the species. In isozyme analyses of 14 *T. hamatum* isolates from different regions of the United States of America, Stasz *et al.* (1989) found three strains strongly related to *Trichoderma (Gliocladium) virens*. The existence of considerable intraspecific variation in DNA patterns was published by Zimand *et al.* (1994) and Turoczi *et al.* (1996). Molecular analysis of *T. hamatum* isolates by Muthumeenakshi (1996) placed them in two distinct ITS-1 type groups which were divided into three RFLP groups. *T. hamatum* RFLP groups 1 and 2 shared an identical ITS sequence and the neotype belonged to this group. *T. hamatum* RFLP group 3 consisted of isolates that originated mainly from mushroom compost. All three groups are closely related to the *T. viride* complex (sect. *Trichoderma*). Muthumeenakshi (1996) also noted that some of the well-identified *T. hamatum* strains are totally different to these groups and are closely related to the *T. harzianum* complex and to certain *T. polysporum* strains. According to Kuhls *et al.* (1997), who sequenced the ITS-1 and ITS-2 regions of the neotype strain of *T. hamatum* (DAOM 167057) and additional *T. hamatum* isolates, the former is similar to species of sect. *Trichoderma*. Sequence differences between the neotype and the remaining *T. hamatum* strains were as high as noted for species belonging to different sections of the genus *Trichoderma*. Recently, Kindermann *et al.* (1998) were using molecular approaches in the investigation of an extensive collection of *ex-type* strains of species related to sect. *Trichoderma* and *Pachybasium* including representatives of all species derived from isolates previously named *T. hamatum* (Bissett, 1991b,c). Sequences of the ITS-1 rDNA revealed three main groups comprising, respectively, (1) sect. *Trichoderma* with two subclades viz. *T. viride/T. atroviride/T. koningii/T. hamatum;* and *T. polysporum/T. piluliferum/T. minutisporum;* (2) sect. *Pachybasium* with *T. harzianum/T. virens* and most of the species derived from the former *T. hamatum* isolates (Kindermann *et al.,* 1998).

The results from all molecular data published so far contrast with the concept of sect. *Pachybasium* of Bissett (defining *T. hamatum* as type species) based on morphological characters. This on one hand emphasizes the importance of molecular approaches in the phylogenetic analysis of *Trichoderma,* but, moreover, it clearly demonstrates that sometimes a third, independently derived set of data is required for an objective solution of phylogenetical problems.

Subgroups of T. viride According to Domsch *et al.* (1980) some strains of the *T. viride* aggregate species produce antibiotics that may confer upon them a potential for biocontrol. The characteristic coconut odour of the species currently attributed to an antifungal pyrone (Claydon *et al.,* 1987) and used as a taxonomic criterion by Rifai (1969) is noted also for some strains of *Hypocrea rufa* (Webster, 1964) and for part of *T. harzianum* and *T. hamatum* (Okuda *et al.,* 1982).

Meyer and Plaskowitz (1989) described two different types of conidial ornamentation among the *T. viride* strains they studied. In the mtDNA studies, Meyer (1991) found two main restriction patterns corresponding to the conidial types. This is in agreement with current studies of Fujimori and Okuda (1994), Arisan-Atac *et al.* (1995), Turoczi *et al.* (1996) and Kuhls *et al.* (1997). Sequence analyses by Muthumeenakshi (1996) and Kuhls *et al.* (1997) demonstrated that *T. harzianum* group 3 according to Muthumeenakshi, *T. koningii* and *T. atroviride* are very closely related to *T. viride* and that the genetic distance between these four taxa is not high enough to classify them into separate species. The lack of type isolates/material for *T. viride* and *T. koningii* is an obvious issue to be addressed in the near future. The description of a *T. koningii* neotype based on morphological as well as molecular features is in progress (Lieckfeldt *et al.,* 1998). The morphology of *T. viride* also needs to be redefined and taxonomic status and names are to be reconsidered.

Hypocrea *and* Trichoderma, *teleomorph-anamorph relationship*

Most known anamorphs of *Hypocrea* species are in *Trichoderma.* The variation within one undoubted *Hypocrea* species can provide information about the amount of variation that can be expected in any *Trichoderma* species; this variation is a key to a species concept in *Trichoderma*. More than 70 species of *Hypocrea* and related genera have been morphologically studied together with their anamorphs by Doi (1967, 1969, 1972, 1975).

Recent molecular genetic studies on *Trichoderma* taxonomy include comparisons of *Trichoderma* strains derived from the teleomorph *Hypocrea* with anamorphic species of Bissett's sections (Kuhls *et al.,* 1997; Leuchtmann *et al.,* 1996; Samuels *et al.,* 1994; Turner *et al.,* 1996). Results of these comparisons are discussed here.

H. schweinitzii *and species of sect.* Longibrachiatum The anamorph of *Hypocrea schweinitzii* is morphologically consistent with sect. *Longibrachiatum* Bissett. Isozyme (Leuchtmann *et al.,* 1996) and rDNA sequence studies (Kuhls *et al.*, 1997) of a comprehensive strain collection of the cosmopolitan species *H. schweinitzii* revealed at least five groups with sequence variation at interspecies level within sect. *Longibrachiatum.* The anamorphs of three groups of *H. schweinitzii* strains could be assigned to, respectively, *T. longibrachiatum, T. citrinoviride* and *T. pseudokoningii*. Isozyme studies of Leuchtmann *et al.* (1996) and RAPD analyses of Turner *et al.* (1996)

support these findings with respect to *T. citrinoviride* and *T. pseudokoningii*. So far, no teleomorph could be linked either to *T. parceramosum* or *T. saturnisporum*.

The H. jecorina-T. reesei *relationship* Previously, Kuhls *et al.* (1996) have shown that the anamorph of the ascomycete *H. jecorina,* morphologically assigned to sect. *Longibrachiatum,* is identical in its ITS sequence to *Trichoderma reesei,* and well distinguished from others in this section. Furthermore, the range of polymorphism in PCR fingerprinting between *T. reesei* and *H. jecorina* was found well within the limits of intraspecies variation. Strains of both species are characterized equally by high cellulase production (Kubicek *et al.,* 1996). Based on these findings, *T. reesei* is the anamorph of *H. jecorina*. Differences in phenotypic characters and isozyme patterns between *H. jecorina* and *T. reesei* (Samuels *et al.,* 1994) indicating certain genetic variability are possibly manifestations of minor mutations occurring during or after the derivation of *T. reesei* from a single population of *H. jecorina* (Kuhls *et al.,* 1996). The *T. reesei/H. jecorina* relationship is the first report of an anamorph-teleomorph connection at the species level that is based on molecular data.

Relationship between other Hypocrea *and* Trichoderma *species* Anamorphic isolates derived from *Hypocrea* species have not generally been used in revisions of taxonomy of *Trichoderma* despite the fact that strains considered by Rifai (1969) to be typical of several of his species aggregates were derived from *Hypocrea* species *(T. piluliferum, T. aureoviride, T. hamatum, T. pseudokoningii)*. Molecular analysis of a larger set of *Hypocrea* species in connection with the studies of sect. *Pachybasium* and *Trichoderma* is in progress in our laboratories and in the laboratory of C.P. Kubicek. Early data have been published by Kuhls *et al.* (1997) as part of a more extensive study of *Trichoderma* sect. *Longibrachiatum.* There is support from ITS sequence data that *H. cf. rufa* strains are identical to *T. viride,* as indicated within the last century from morphological observations (see Webster, 1964) and more recently by RAPD analysis (Arisan-Atac *et al.,* 1995).

2.3.2 Species concept of Gliocladium

Like *Trichoderma, Gliocladium* is a genus of hyphomycetes. Anamorphs with *Gliocladium* morphology are found in at least six hypocrealean genera: *Hypocrea* (Webster, 1964), *Nectria* (Samuels, 1976), *Nectriopsis* (Samuels, 1973), *Roumegueriella* (Bainier, 1910), *Sarawakus* (Samuels and Rossmann, 1992) and *Sphaerostilbella* (Seifert, 1985). Although species of *Hypomyces* are reported to have *Gliocladium* anamorphs (Petch, 1938), these species are now considered to belong to *Sphaerostilbella* (Seifert, 1985). The diversity of these relationships clearly indicates that *Gliocladium* is polyphyletic. Focusing particularly on the type species, *G. penicillioides,* and two species with biocontrol properties, *G. virens* and *G. roseum,* Rehner and Samuels (1994) have constructed a gene phylogeny based on sequence data of the 5' region of the nuclear 28S large subunit rRNA from different hypocrealean anamorphs (Figure 2.2). Results show that all three *Gliocladium* species represent phylogenetically distinct groups within the *Hypocreales* and confirm the hypothesis of the authors that *G. virens, G. roseum* and *G. penicillioides* are generically distinct. *G. roseum* forms a group with species of *Nectria* and is excluded from *Gliocladium* and *Trichoderma,* whereas *G. virens* is derived from within *Hypocrea* with anamorphs in *Trichoderma*. The type species *G. penicillioides* (anamorph of

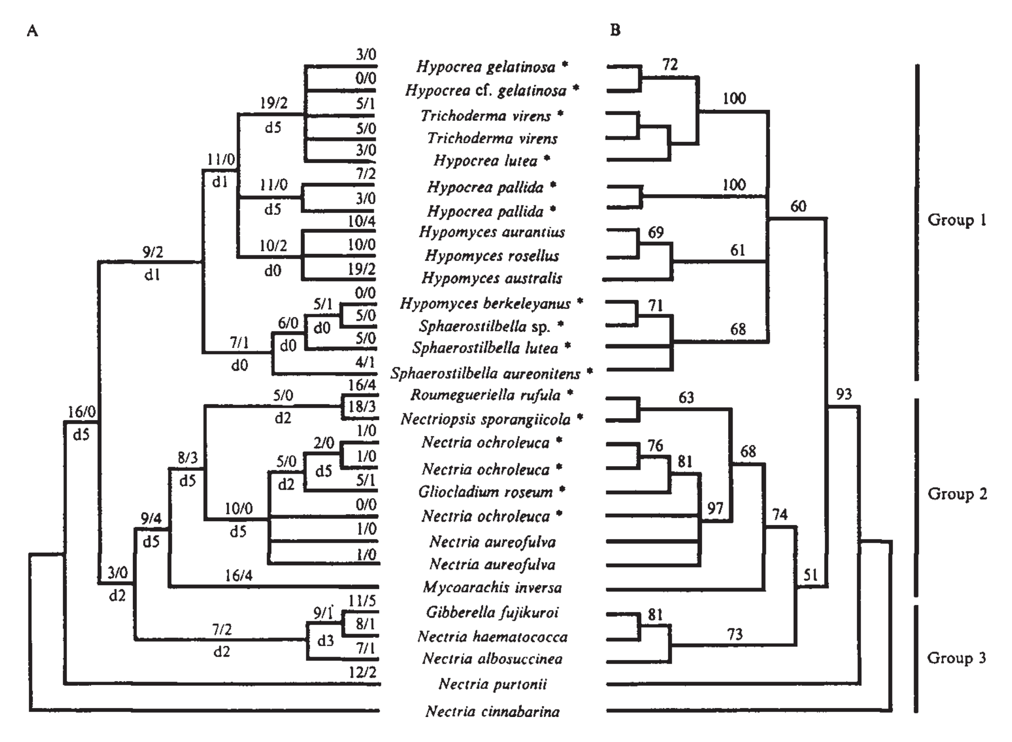


Figure 2.2 Phylogeny of *Gliocladium* analyzed from nuclear subunit rDNA sequences (reproduced with permission from Rehner and Samuels, 1994). Cladograms are inferred by maximum parsimony analysis. (A) Strict consensus of 56 shortest trees of 320 steps. Branch lengths are indicated above the branches and the number of characters unequivocally supporting each branch are separated by a /. Decay indices are indicated below branches. (B) Bootstrap parsimony analysis with bootstrap intervals from 1000 replications and nodes supported in >50% of bootstrap replicates are indicated. Species whose anamorphs have a *Gliocladium*-like morphology are indicated by asterisks. *Hypomyces berkeleyanus* was renamed into *Sphaerostilbella berkeleyanus*.

Sphaerostilbella aureonitens) is grouped together with other *Sphaerostilbella* species in a monophyletic clade. The DNA data support Seifert's (1985) hypothesis that *Sphaerostilbella* is monophyletic with respect to unique morphological characters. Rehner and Samuels (1994) suggest a restriction of *Gliocladium sensu stricto* to the anamorphs of *Sphaerostilbella*. However, the anamorphs of one group of *Hypocrea* species that includes *H. pallida* cannot be distinguished from the morphological concept of *Gliocladium sensu stricto* despite the fact that these *Hypocrea* species differ from *Sphaerostilbella* in their large subunit sequences (Rehner and Samuels, 1994). While these *Hypocrea* species can be easily removed from *Hypocrea,* there is strong biological and morphological similarity to *Sphaerostilbella* and neither teleomorph genus could be predicted on the basis of the *Gliocladium* anamorph alone. These observations raise important questions about the advisability of abandoning classification of mitotic fungi on the basis of form (Gams, 1995).

2.3.3 Boundary between Trichoderma *and* Gliocladium

Rifai (1969) differentiated *Trichoderma* from *Gliocladium* primarily by the angle at which branches and phialides were borne and referred the species *T. virens* to

Gliocladium. von Arx (1987) as well as Bissett (1991a,b) included the species in *Trichoderma*. According to Bissett (1991b), *T. virens* shares cultural and morphological characters with typical *Trichoderma* species of sect. *Pachybasium* and bears little resemblance to *G. penicillioides* or *G. roseum. Trichoderma virens* has no known teleomorph, but it is morphologically similar to the anamorph of *H. gelatinosa* (Bissett, 1991b; Webster, 1964) and both species were included in *Trichoderma* sect. *Pachybasium* by Bissett (1991b).

Molecular data also support the inclusion of *G. virens* in *Trichoderma* (Rehner and Samuels, 1994). In the 28S rRNA sequence studies of Rehner and Samuels (1994), *Hypocrea lutea, H. gelatinosa* and *T. virens* form one well-supported monophyletic group distinct from the remaining *Gliocladium* species and related anamorphs.

A possible relation of *T. virens* to sect. *Pachybasium* Bissett is indicated from sequence similarities of an endochitinase gene in *T. virens* and *T. harzianum* (Hayes *et al.,* 1995). The *ex-type* isolate of *G. virens* CBS 249.59 along with five other strains of *G. virens* which are used as biocontrol agents were characterized using molecular markers (Muthumeenakshi, 1996). The ITS sequence of these isolates revealed that *G. virens* is very closely related (97% similarity) to *T. harzianum* group 1. This conclusively proved that this is a species of *Trichoderma*.

Two more *Gliocladium* species are also found to be related to *Trichoderma.* The *ex-type* isolate of *G. flavofuscum* is similar to *T. virens* in all molecular markers. This confirms the findings by Bissett (1991c) who transferred *T. flavofuscum* comb. nov. from *Gliocladium* to *Trichoderma* section *Pachybasium* due to morphological features which ally this species with *T. virens* and *T. crassum*. The hypothesis of supposed conspecificity of *T. virens* and *T. flavofuscum* as indicated by molecular data needs to be verified. A strain of *G. viride* (CBS 228.48) was found to be related to *T. polysporum* complex in ITS sequence and it revealed its close relatedness to *Trichoderma* in all molecular markers studied (Muthumeenakshi, 1996).

2.4 Future prospects—a critical evaluation of taxon definition based on molecular data

"The more extensive and complex the involvement of *Trichoderma* and *Gliocladium* in biocontrol, the more useful and necessary is their accurate classification", Papavizas pointed out in 1985. The state of the science twelve years later is reflected by the existence of a considerable amount of molecular data characterizing morphologically assigned species of both genera in more detail. Molecular techniques allow a rapid and reliable identification of *Trichoderma* species and strains. In general, molecular data confirm the morphological classification of *Trichoderma;* in some cases a revision is suggested.

The basic task of taxonomy is to be the recognition of the limits of an individual species, regardless of methodology. Molecular data do provide a more objective measure of the genetic variability of individuals than phenotypic characters do, but it remains difficult to interpret the phylogenetic and taxonomic significance of the observed similarity. Another problem is the amount of molecular data needed for significant phylogenetic conclusions. Classification should not be based on single characters (Seifert *et al.,* 1995). Therefore, the combination of sequence analysis of conserved rDNA, analysis of genomic DNA by fingerprinting/RAPDs

and analysis of mitochondrial DNA seems to be the most appropriate way to resolve species complexes as demonstrated for *Trichoderma*.

Taxonomic stability is urgently needed in the face of the large amount of biological and biotechnological research on *Trichoderma*. The following is an attempt to discuss the molecular data with respect to the concept of species in *Trichoderma* (Figure 2.3):

(1) Identical 5.8S rRNA and less variable ITS-1 and ITS-2 sequences for the investigated species of the sections *Longibrachiatum, Pachybasium, Trichoderma, Saturnisporum* and *Hypocreanum* (Kuhls *et al.,* 1996; Muthumeenakshi, 1996) confirm the close relatedness of all species within *Trichoderma* and indicate that the taxonomy of the genus may be less complex than expected from morphological data.

(2) How many species are included in sect. *Longibrachiatum?* Low molecular divergence could support the recognition of one species—*T. longibrachiatum*—with several subspecies. Is *T. saturnisporum* with its tuberculate conidia another subspecies of this species? Or if *Hypocrea schweinitzii* (sect. *Longibrachiatum*) represents more than one species, as indicated by at least five distinct groups of sequence divergence comparable to the interspecies variation within sect. *Longibrachiatum,* is it likely that the anamorph of each group is a distinct species of *Trichoderma?*

    The species concept taken into consideration determines how to evaluate measured genetic differences. Following the definitions of Cracraft (1983) and Wheeler and Nixon (1990), the phylogenetic species concept could result in an increasing number of species, because the concept has no limit to diagnosability of unique character states.

(3) When is there enough molecular evidence to postulate a teleomorph-anamorph relationship? Can identical rRNA ITS sequences and "high" (>70%) similarities in fingerprinting/RAPD patterns serve as sufficient criteria? At present, this seems to be the best approach to integrate anamorphic fungi into the teleomorphic fungal system and to create a truly holomorphic classification system.

(4) A problem in the study of molecular taxonomy of *Trichoderma* sections *Pachybasium* and *Trichoderma* is the unavailability of *ex-type* strains of most of the species. There are numerous soil isolates grouped according to biochemical and genetic properties, but the exact relation to a certain morphologically defined species is lacking. Designation of neotypes is necessary for *T. harzianum* and *T. koningii*. For *T. viride,* a recent specimen has to be related to the historical type material (herbarium specimen).

(5) The measured heterogeneity of species in sections *Trichoderma* and *Pachybasium* on one hand and the overlapping of both sections on the other hand strongly suggests the need for improvement of morphologically based taxonomic systems. It seems likely that these fungi are evolving rapidly but that evolution is manifested primarily in the genomes *per se* rather than in morphology.

If it is common that even sectional affinities cannot be predicted from morphology, it is obvious that taxonomic revision of *Trichoderma* will be even more difficult than was currently thought.

In our opinion, the aim is to provide a means to identify organisms and to predict biological properties/activities based upon phylogenetic relationships; this

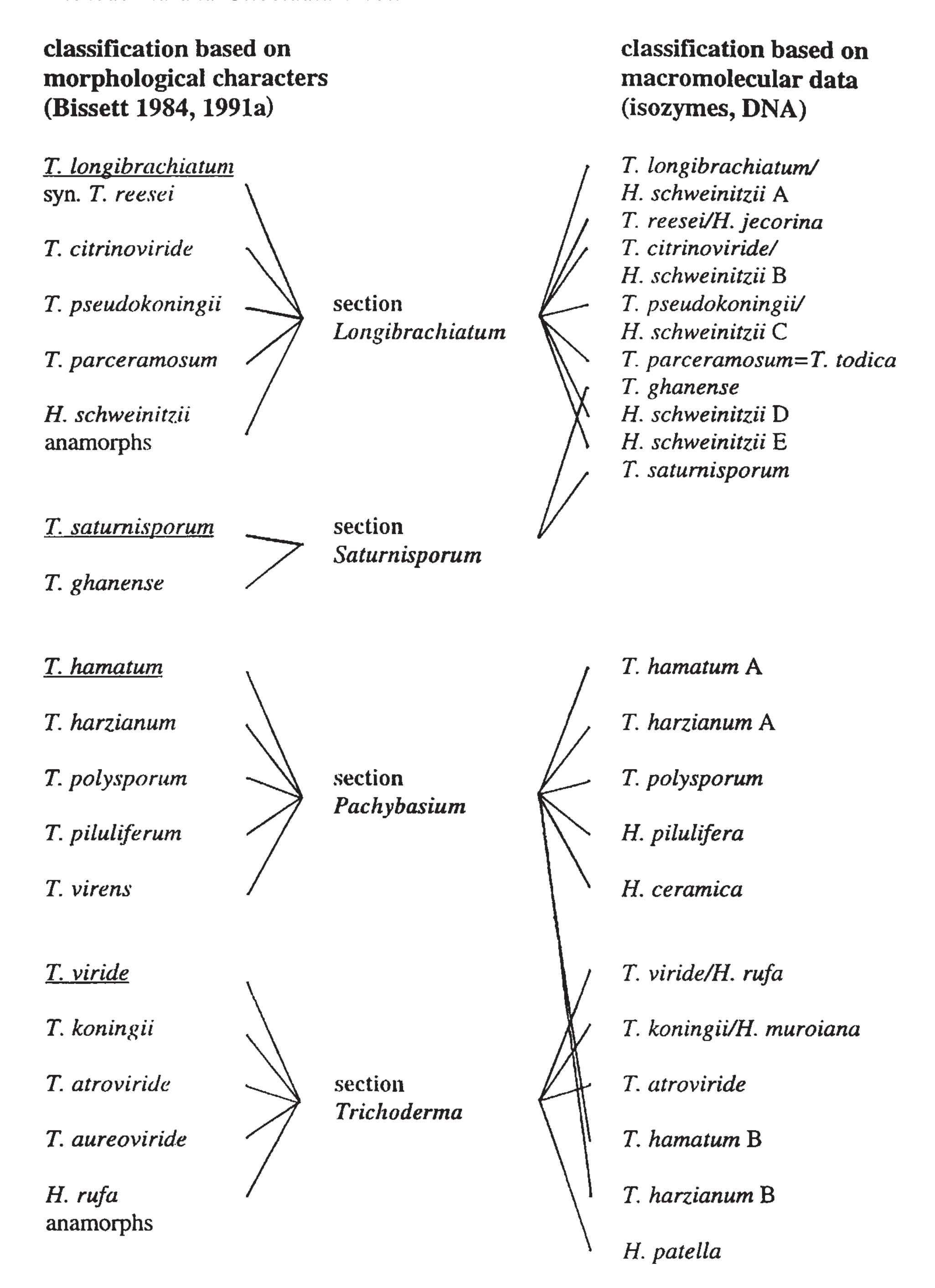


Figure 2.3 Comparison of the classification of the genus *Trichoderma* according to isozyme and DNA data with the morphologically based taxonomy proposed by Bissett (1984, 1991a–c).

includes the reconstruction of phylogenetic history. The best way to a reconstruction of phylogenetic history is the investigation of nucleic acids. In fungi, molecular analyses based on nucleic acids have made possible the link between individual asexual species of the *Fungi imperfecti* and sexual species of the *Ascomycetes* (e.g., Samuels and Seifert, 1995). For *Trichoderma,* Kuhls *et al.* (1996) have demonstrated this possibility at the species level for the individual anamorph/teleomorph pair *T. reesei/H. jecorina*. A combined investigation by morphological and molecular approaches of the asexual *Trichoderma* and the sexual *Hypocrea* is the key to a phylogenetic species concept of the genus *Trichoderma*.

According to all data summarized in this chapter, there is strong evidence that the genus *Trichoderma,* viewed phylogenetically, comprises a very young and rapidly evolving genus with many intermediate forms making the whole genus a continuum of development. The more isolates included in the analyses the more the borders between morphologically defined species will disappear resulting in a small number of "real" species forming the genus.

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3

Ecology of *Trichoderma*

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3.1 Introduction

Trichoderma and *Gliocladium* are ubiquitous soil dwellers. They sporulate profusely and hence are widely recorded in all manners of surveys. These general saprophytes have minimal nutritional requirements and can grow rapidly, besides producing a scintillating array of secondary metabolites. Additionally, they have the ability to transform an extraordinarily wide variety of organic materials of both natural and xenobiotic origin. They are well-known hyper-producers of degradative enzymes including chitinases involved in lysis of fungal mycelia (Volume 2, Chapter 7) and cellulases (Volume 2, Chapter 1). With such virtues, it is perhaps not surprising that they appear as adept colonizers and have been promoted indirectly as biocontrol agents (Volume 2, Chapters 8–10) and plant growth stimulators (Volume 2, Chapter 6), besides being noxious pests of the mushroom industry (Volume 2, Chapter 11). There are also periodic reports of *Trichoderma* causing infections of humans (Loeppky *et al.,* 1983) and attacks on immunocompromised patients (Seguin *et al.,* 1995). In spite of their wide occurrence and general importance, relatively few reviews address their ecology (Eveleigh, 1985; Papavizas, 1985; Samuels, 1996). This chapter attempts to set their ecological status in general perspective. The taxonomy, methodology for identifying populations, and general physiology are briefly discussed and then examined in ecological perspective, namely interactions between *Trichoderma,* its environment and other organisms in that environment.

3.1.1 Taxonomy

Persoon (1794) first described the taxonomy of *Trichoderma* in his classification of fungi (Figure 3.1). He did not illustrate his brief narrative on *Trichoderma,* which was unfortunate because his keen powers of observation yielded clearly recognizable illustrations of other fungi including *Ascobolus, Mucor* and *Puccinia,* besides some slime molds, *Physarum, Stemonitis* and *Trichia*. The ubiquitous nature of *Trichoderma* was recognized early as it was a prominent component of soil fungal

Trichoderma (hair; skin)
Pulvere farinaceo tegmine tomentoso cincto.
Characterized by a mealy powder surrounded by short matted hairs
T. viride, subrotundum effusumque, pulvere viridi, villo albido.
Pyrenium *lignorum* Tode var. α.
T. viride (green), slightly round and diffuse, greenish powder and with white woolly hairs
T. caesium, subeffusum, pulvere nigrescente, villo cæfio.
Prov. ad trunc. aridos. (Fraxin. excels., querc.)
T. caesium (bluish-gray), slightly diffuse, with a blackish powder (and) blue hairs. Collected from dry tree trunks (ash and oak).
T. aureum, subrotundum, totum, aureum. (Pyren. lign. T. var. β.)*
T. aureum (golden), slightly round, entirely golden
T. roseum, majusculum subrotundum roseum.
T. roseum (pink), somewhat large, slightly round, pink.

Figure 3.1 The first description of the mold *Trichoderma* (from Persoon, 1794)

populations based on dilution plate counts. By the mid-1920s, Abbott (1926–27) was describing four fairly well-defined groups [*T. glaucum, T. koningii,* and two variant forms of *T. lignorum*] and recorded that they "occurred with considerable regularity" in diverse American and foreign soils.

In reality, the speciation is far more complex. Persoon had rather hazily described four *Trichoderma* species and did not realize that he was describing only the anamorphic state. Not until over half a century later was the relationship between *Trichoderma viride* and its teleomorph *Hypocrea rufa* clarified by Tulasne (1860) and clearly drawn in the Tulasnes' monograph five years later (Tulasne and Tulasne, 1865). By 1902 this correlation was fully accepted (Smith, 1902).

To ably distinguish the several *Trichoderma* anamorphic species requires considerable taxonomic expertise. Indeed, Papavizas interpreted this status by noting that prior to Rifai's classification (1969), all citations that he discussed (Papavizas, 1985) should be considered at "the genus level only". Even after that date, from a perusal of biochemical catalogues selling cellulases, it appears that this taxonomic complexity has been "simplified" and sometimes resulted in any green-spored *Trichoderma*-like mold being indiscriminantly accorded the name *T. viride*. Rifai's (1969) classification of *Trichoderma* based on nine "species aggregates" was a very practical advance. Since then the classification of *Trichoderma* species has continued to evolve (see the studies of Doi, Domsch, Gams and Bissett reviewed in Volume 1, Chapter 1). Today, the taxonomy of *Trichoderma* and *Gliocladium,* based on use of a full armamentarium of classical, biochemical and molecular biological tools, is on a fairly firm basis, although it still requires considerable expertise to distinguish the species (Volume 1, Chapters 1 and 2).

3.1.2 Enumeration of populations

Trichoderma spp. are generally fast growing, and simple dilution plating of soil routinely yields positive results. Typical descriptions of soil mycobiota record the presence of rapidly growing and profusely sporing forms with Aspergilli, Penicillia, Fusaria and *Trichoderma* spp. forming the dominant populations. Isolation of *Trichoderma*

species is discussed in depth in Volume 1, Chapter 1. With *Trichoderma's* proclivity for conidiation, it is not surprising that it is reproducibly recovered in all manners of sampling—from air (Bokhary and Parvez, 1995; Takatori *et al.,* 1994), from seeds (Smith, 1902), from dead plant materials and even from ant and termite frass (Fisher *et al.,* 1996; Sreerama and Veerabhadrappa, 1993). However, colony-forming units (CFU) obtained through direct soil plating probably reflect the numbers of conidia laying dormant in the soil, rather than active mycelial mass. As the spores are so numerous, even washing of the samples may remove only an insignificant proportion of the conidiospores (Warcup 1950, 1960). For instance, 99% removal could still result in the majority of CFU colonies arising from conidia rather than mycelium. Waksman (1916), in his MS thesis studies showing the wide occurrence of *Trichoderma* spp. from soils from disparate geographical locations, used soil particles as inocula in attempting to obtain colonies arising from active mycelium rather than from spores. Whether or not this technique results in recovery of cultures only from active mycelium is undecided as spore germination and subsequent growth can be extremely rapid. Using recently developed techniques, it is now possible to make correlations between the results of the dilution plate method with those methods that specifically assess the presence of mycelium or of conidiospores by use of specifically tagged antibodies (see below).

3.1.3 General physiology

Trichoderma and *Gliocladium* species are metabolically versatile yet are also eclectic. Nitrogen sources are generally well utilized from ammonium compounds to amino acids (L-alanine, L-aspartate, L-glutamic acids) to proteins, although nitrate assimilation is often poor and is species dependent (Danielson and Davey, 1973c). Carbon substrates are extremely diverse. *Trichoderma* species are one of the few groups of organisms that can metabolize C_1 compounds illustrated by the growth of *T. lignorum* on methanol (Tye and Willets, 1977). Several *Trichoderma* strains degrade hydrocarbons (Davies and Westlake, 1979), and this ability is perhaps reflected as they form major components of fungal populations from soils polluted with oil (Gudin and Chater, 1977; Llanos and Kjøller, 1976; Pinholt *et al.,* 1979). The more classical carbon substrates include a range of general sugars, though there is considerable variation among species in their abilities to utilize inulin, melezitose, raffinose, sucrose, besides tannic and gallic acids (Danielson and Davey, 1973c). Rhamnose, inositol and *a*-methyl-D-glucoside are exceptions and are poorly used. The utilization of sucrose, raffinose, melezitose and nitrate, and the reactions toward tannic and gallic acids, has been suggested to be used as an aid in classification. *Trichoderma* and *Gliocladium* species also attack a variety of polysaccharides including cellulose, chitin, laminaran, pectin, starch and xylan and secrete massive amounts of the respective polysaccharases (section 3.3).

The biochemical versatility of *Trichoderma* species is further illustrated by their ability to transform an array of complex plant materials (indole alkaloids and vanillin—Kieslich, 1976; *Aspergillus* aflatoxin—Mann and Rehm, 1976), besides xenobiotic pesticides including Arachlor, DDT, Aldrin, Dieldrin, Malathion and Dalapon. Conversely, their synthesis of a diverse range of metabolically active secondary metabolites is quite remarkable (Volume 1, Chapter 7). Such compounds clearly affect other organisms in the *Trichoderma* ecological niche.

In retrospect, with the ability to utilize such a variety of substrates and to effect such diverse transformations in combination with being able to survive under relatively adverse conditions, it is not too surprising that *Trichoderma* spp. are associated with the spoilage of paintings and masonry besides the deterioration of rubber and plasticizers (Pitt, 1981; Rose, 1981).

It is manifest that *Trichoderma* and *Gliocladium* are wondrously endowed in being able to attack diverse substrates including other fungi, their ability to compete for nutrients, and their production of antibiotics. They use these capabilities interactively to facilitate their development in particular habitats.

3.2 Methodology

3.2.1 Characterization of Trichoderma populations

A central ecological tenet is to understand the status of a population, its abundance and activity and its survival, in brief its total temporal development. It is also said that ecology is the study of biology under the worst possible conditions. This is critical, and from this perspective the following discussion on methodology is presented. For determination of *Trichoderma* and *Gliocladium* populations, the primary approach has been through soil dilution plating. Factors affecting this methodology include the nature of culture media used to optimize the recovery of strains (for greater depth see Volume 1, Chapter 1), the effects of physical factors such as temperature on the recovery of soil populations, besides the use of modern approaches including the application of monoclonal antibodies and of molecular biological methodology in the identification of *Trichoderma* mycelium and spores.

3.2.2 Assessment of populations (selective media)

The basic dilution/plating technique appears generally sufficient to isolate *Trichoderma* spp. because most species grow rapidly and their spores are abundant. Indeed, they often out compete other microbes, and in order to isolate slower-growing fungi, inhibitors are used to slow the development of *Trichoderma* spp. For instance, lithium chloride (0.6%) selectively inhibits their germination and retards their mycelial growth (Wildman, 1991). Recoveries of *Trichoderma* and *Gliocladium* based on soil dilution plating protocols have clearly indicated their ubiquitous status in soils worldwide, even though emphasizing the ability of these fungi to profusely conidiate.

Quantitative isolation of *Trichoderma* has been approached through the development of selective culture media. The archetypal concoctions employ chemicals and dyes such as rose bengal, crystal violet, oxgall (2-deoxycholate) and pentachloronitrobenzene (PCNB) in combination with various fungicides. Analogous inhibitors such as allyl alcohol in combination with vinclozolin have been usefully employed (Davet, 1979). The classic recovery medium is TSM (*Trichoderma* Selective Medium) (Elad *et al.,* 1981), later improved to restrict the development of Fusaria by the incorporation of benomyl for strains resistant to this fungicide (Elad and Chet, 1983). A range of alternate fungicides has been usefully employed. For instance, promamocarb or metalaxyl were successfully substituted for the fenaminosulf that was originally included to inhibit Oomycetes (Askew and Laing, 1993). Other

media have been developed to circumvent the use of the general inhibitors rose bengal and PCNB though finally using an alklyaryl polyether alcohol to obtain optimal results (Papavizas and Lumsden, 1982). In principle, it has been the use of narrow spectrum inhibitors active towards other dominant fungi (e.g. Fusaria and Oomycetes) that has aided the quantitative recovery of *Trichoderma* and *Gliocladium*.

3.2.3 Temperature

The optimum growth temperature for most *Trichoderma* spp., as judged from published laboratory studies, is in the 25–30°C range. However, most isolates were originally selected at room temperature perhaps thereby excluding the recovery of slow growing psychrophilic and thermotolerant forms. Indeed temperature can be highly significant in the recovery of *Trichoderma* species. Roiger *et al.* (1991) recovered *T. virens, T. harzianum, T. koningii* and *T. hamatum* through incubation at 8–24°C, with fewer overall colony-forming units at 8°C. *T. viride* was only recovered at 8 and 16°C, perhaps suggesting a seasonal niche.

Further distinction is apparent as illustrated when *Trichoderma* species were grown competitively in mixed culture at different temperatures (Widden and Scattolin, 1988). Using a model spruce-needle substrate, the development of five *Trichoderma* species was compared with regard to colonization and to their succession of substrates that had been previously colonized by other *Trichoderma* spp. *T. hamatum* and *T. koningii* grew fastest at 25°C and displaced other species, while *T. viride* and *T. polysporum* showed greater competitiveness at lower temperatures.

Clearly temperature is critical in the qualitative and quantitative recovery of *Trichoderma* and *Gliocladium* and is a determinant affecting their colonization. It is necessary to employ a range of incubation temperatures in order to optimize the assessment of the populations *per se,* and also in relation to seasonality (Widden, 1981; Widden and Arbitol, 1980).

3.2.4 Isozymes

The recovered *Trichoderma* isolates can be identified, strains differentiated, and even cladistic analyses developed (Volume 1, Chapter 2) by use of isozyme characterization. Isozymes are distinct enzymes yet with the same activity and can be distinguished through their electrophoretic profiles. Stasz *et al.* (1988) used isozyme profiling of 63 enzymes with six species of *Trichoderma* and *Gliocladium* (25 strains), and were able to resolve species groups. This study was successfully extended for cladistic analysis using 16 enzymes from 71 strains (Stasz *et al.,* 1989). An additional interesting comment was that “wide distribution of numerous alleles among morphologic species suggests that extensive genetic exchange occur among these taxa” (Stasz *et al.,* 1989). These studies of isozymes can also be used to imply a general indication of the ecologic niche of the organism; for example, a rich variety of degradative enzymes might well suggest a niche of a saprobe.

3.2.5 Monoclonal antibodies

Tagged monoclonal antibodies (MAb) are being developed as rapid, highly specific tools to identify *Trichoderma;* for example the development of an enzyme-linked

immunosorbent assay (ELISA) specific to most species of *Trichoderma* and to related anamorphic *Hypocrea* species (Thornton *et al.,* 1994). This MAb does not cross-react with common (non-*Trichoderma*) soil fungi. It is of particular interest with regard to assessing active biomass, as it only binds to live *Trichoderma/ Hypocrea* mycelium and not to senescent conidia nor to senescent chlamydospores. In a further approach, Thornton and Dewey (1996) developed a monoclonal antibody active towards phialoconidia. This MAb is highly specific, recognizing only the phialoconidial stage of *T. harzianum* strain T95 (against which it was raised), three of five other *T. harzianum* strains, *Trichoderma viride* (2 of 3 strains), and one of two strains of *T. koningii*. Although this antibody cross-reacts with conidia of at least one *non-Trichoderma* fungus *(Thielaviopsis basicola),* its general specificity allows the determination of *Trichoderma* conidial biomass as opposed to mycelium or chlamydospores.

Monoclonal antibodies may also have taxonomic/phylogenetic utility with *Trichoderma*. Phylogenetic insights may develop from noting the cross-reactivity of the antibody towards *T. harzianum* strain T95 phialoconidia with the spores of *T. viride* for which there are also some microscopic and macroscopic phenotypic similarities (Thornton and Dewey, 1996). It will be of interest in the future to determine the genetic basis of this cross-reactivity. In a further application, variations in the mycelial antibody recognition within different isolates of the "same" *Trichoderma* species were later attributed to misassignment of the original *Trichoderma* cultures (Thornton *et al.,* 1994). A qualifying aspect is that although analysis of DNA patterns of *T. koningii* strains suggested a close affinity to a strain of *T. harzianum,* this particular strain was not recognized by the antibody (Thornton and Dewey, 1996).

3.2.6 Uses of DNA

The most powerful new methods of ecology are those based on the use of unique DNA sequences, for what is more indicative of a population or of a species than its common genetic heritage? As DNA methods have evolved, less and less starting material is required, which increases the method's usefulness. Indeed, with polymerase chain reaction (PCR) DNA amplification protocols (Saiki *et al.,* 1988), it is possible to analyze the DNA of 1–10 fungal spores (Lee and Taylor, 1990). The approaches are briefly reviewed for the sake of continuity, but are more fully detailed in Volume 1, Chapter 2.

DNA for identification

Today, DNA methods are commonly used for identification and phylogenetic classification (Volume 1, Chapter 2). Typically, the actual DNA sequences of one or more genes are needed for phylogenetic analyses (Kuhls *et al.,* 1996; Rehner and Samuels, 1994). Data produced with RAPD (Randomly Amplified Polymorphic DNA) (Welsh and McClelland, 1990) and other methods of "PCR fingerprinting" may be analyzed for phylogenetic purposes (Meyer *et al.,* 1992) but are more commonly used for comparison of isolates. For example, Kuhls *et al.* (1995) compared PCR fingerprints of *Trichoderma ex-type* strains from several culture collections and found that the patterns were identical. Since the patterns for other isolates assigned to the same species differed, this indicates that cultures from the original-type isolate were not

contaminated nor confused during decades of independent culture, and the cultures maintained a high degree of genetic identity over that time. The *Trichoderma* isolates can be distinguished by their fingerprint patterns, and in the words of Kuhls *et al.,* "PCR fingerprinting is recommended as a basic tool for proving the identity of strains, especially with regard to comprehensive culture collections." Indeed so, as in a further joint study, the biogeography of ex-type *Trichoderma* strains has been dramatically illustrated using these methodologies (Turner *et al.,* 1997). The finesse of the methodology was also illustrated in being able to place the *Trichoderma* sect. *Saturnisporum* in the *Trichoderma* sect. *Longibrachatum,* a conclusion supported by ribotyping restriction analysis and also by sequence analysis (Turner *et al.,* 1997).

RAPD fingerprinting was used by Arisan-Atac *et al.* (1995) to identify subgroups of *Trichoderma* capable of chestnut blight biocontrol; they found that the biocontrol activity was spread among several distinct groups and subgroups of *Trichoderma*. Restriction fragment length polymorphisms (RFLP) in combination with RAPD techniques were useful in grouping and distinguishing isolates of *T. harzianum* in mushroom compost (Muthumeenakshi *et al.,* 1994). One of the three groups distinguished was a particularly aggressive colonizer and could be readily tracked using these DNA methods. The exquisite sensitivity of RAPD was also proven by Schlick *et al.* (1994) when they were able to distinguish gamma-ray-induced mutants from their parent *Trichoderma* strains by RAPD. This ability to readily distinguish isolates from nature and to place them within other members of the species and genus is extraordinarily valuable.

DNA for ecological studies

Using recombinant DNA methods such as cloning and knockout mutations, researchers are altering the metabolic capabilities of *Trichoderma* to discover the basis for its biocontrol activities or pathogenicity (Chet and Inbar, 1994; Goldman *et al.,* 1994). For example, greater biocontrol of *Rhizoctonia solani* by *T. harzianum* strains was obtained by increasing the level of prb1 proteinase through cloning (Flores *et al.,* 1997).

Both DNA probes and antibodies can be linked to fluorescent dyes to create probes. It should thus be possible to determine visually the actual proportion of active vs. inactive *Trichoderma* mycelium, or even *Trichoderma* vs. total fungal biomass in an environmental sample, given the correct assortment of probes. Comparable practical results have been achieved with diverse bacterial communities from the rumen (Schofield *et al.,* 1997), activated sludge (Wagner *et al.,* 1993), and environmental water samples (Lim *et al.,* 1996).

3.2.7 Biochemical assessment of ecological niche

A range of methods allows estimation of a microbial population, its biomass and its activity (White, 1983). To this end characteristic fungal components have been quantitated including chitin (from the cell wall), ergosterol (fungal membranes) and even ATP when determined in the presence of bacterial inhibitors such as penicillin and streptomycin. As chitin is specific to the walls of non-Oomycete fungi and it can be used to assess fungal populations in model systems but only if insects and arthropods are excluded. Analysis of chitin *per se* does not distinguish

between walls of dead and living mycelium. The unique fungal sterols are perhaps better indicators of fungal biomass defining the greater mass of mycelium in comparison to that of spores. Enzyme activity (e.g. esterase, chitinase, cellulase) has also been employed to gain some general assessment of microbial activity and such could be immunologically identified following separation (Sreerama and Veerabhadrappa, 1993). This is a useful approach to assess activity, but it must be remembered that enzymes can remain active in the soil long after the producing organism has died. In addition, enzymes will be produced only if proper induction conditions were present when the fungus was actively growing.

Clearly the basic parameters for determination of populations of fungi are in dynamic flux. For representative isolation of active members of such populations, attention must be paid to the recovery medium and the physical environmental factors. The assessments can be considerably improved by the use of modern technologies such as isozyme profiling, monoclonal antibodies and nucleic acid probes. Specific monoclonal antibodies allow identification of *Trichoderma* spp. and even distinguish between metabolically active *Trichoderma/Hypocrea* mycelium and phialospores. Spore counts can be reduced by the Warcup washing protocol. Yet perhaps pretreatment of soil inocula prior to plating with the directed delivery of conidiocides through the use of monoclonal antibody-tagged fungicides would also result in more representative assessments of the abundance of active mycelium. DNA fingerprinting and sequencing allow identification of species and strains. MAbs and nucleic acid probes clearly represent major methodological advances and provide ecologists with vital tools for delving deeper into the many roles and activities of *Trichoderma.*

3.3 General ecology

3.3.1 Habitats

Trichoderma spp. comprise a fast-growing group that appear to be extremely common in agricultural, prairie, forest, salt marsh and desert soils in all climatic zones (Danielson and Davey, 1973a; Domsch *et al.,* 1980, Roiger *et al.,* 1991; Wardle *et al.,* 1993). They are particularly prevalent in the litter of humid, mixed hardwood forests, comprising a minor component of the microbiota in the initial colonization but subsequently becoming more dominant in the H and F horizons. Characteristically they are saprophytes, with the exception that they can attack other fungi. *Trichoderma* spp. can be detected in soils by smell, the coconut odor associated with certain of them being due to the volatile 6-pentyl-a-pyrone (Collins and Halim, 1972; Kikuchi *et al.,* 1974; Moss *et al.,* 1975). Recovery of specific species and estimates of their occurrence are routinely obtained from soil dilution plating. They can constitute up to 3% of the total fungal propagules from a wide range of forest soils (Danielson and Davey, 1973a) and 1.5% of the fungi in pasture soils (Brewer *et al.,* 1971). These are useful assessments. Yet in a different approach through the use of RAPD-based screening even with a limited number of cultures available, considerable insight was gained into the biogeography of *Trichoderma* spp. Turner *et al.* (1997) showed that *T. longibrachiatum* and *T. citrinoviride* overlapped through much of their geographic ranges with *T. longibrachiatum* also occurring in Africa and India, while *T. citrinoviride* occurred in south-east

Asia but not Africa or India. *T. pseudokoningii* (the anamorph of *H. schweinitzii*) occurred only in New Zealand and eastern Australia. Another *H. schweinitzii* subgroup that was homologous to *T. citrinoviride* occurred in the temperate climates of Europe and the USA. The success of these studies was very dependent on the apparent high phenotypic stability, and "the high stability of RAPD characters amongst the populations of wide geographic separation indicates effective clonal isolation…The wide geographic distribution of these characters may thereby be due either to efficient dispersal (wind or insects) or be indicative of a very early evolutionary process" (Turner *et al.,* 1997).

3.3.2 Substrates

As noted in the introduction (3.1.3), *Trichoderma* and *Gliocladium* spp. are remarkable for the diversity of substrates that they can metabolize. In forest soils with the abundance of biomass, it is somewhat enigmatic that in spite of the marked cellulolytic nature of most *Trichoderma* species (Volume 2, Chapter 1), their ability to degrade natural woods (lignocellulose) is relatively weak. In pure culture studies, several *Trichoderma* species were relatively ineffective in degrading dogwood leaves and loblolly pine needles (Danielson and Davey, 1973c) and beechwood (Butcher, 1968). Strains can colonize wood by using non-structural carbohydrates. For instance, in attack of loblolly pine logs only the ray parenchymatous cells and the half-bordered pits were destroyed (Hulme and Stranks, 1970). In this sense they have been classified as soft-rot fungi, which modify the wood but do not dramatically degrade it. These actions can be put to good use by opening up the wood thereby facilitating enhanced penetration, thus improving application of timber preservatives (Johnson and Gjovik, 1970). However, the attack is species "host-specific", and with Douglas fir no attack was found. *Trichoderma* and *Gliocladium* spp. have also been used to prevent the entry of white and brown rots into commercial woods by spraying the cut ends of stacked timber and allowing them to develop a protective mantle over the cut base. The major rationale for this protective action is that *Trichoderma* and *Gliocladium* rapidly removed non-structural carbohydrates (sugars and starch) which then limits the attack by secondary invaders, including both wood rots and also blue stain fungi. However, under natural circumstances they appear to be secondary invaders. *T. harzianum* has been shown to inhibit wood rots through production of volatile antibiotics (Morrell, 1990).

Rossman (1996) in noting the ecological niche of *Trichoderma* and *Gliocladium* spp. in partially decayed wood, suggested a novel rationale for their development. She proposed that these fungi developed in this niche, not principally as a result of their wood degrading properties but through their necrotrophic action towards the true wood rot fungi. It is noteworthy that *Trichoderma* spp. are a source of commercial cell wall lytic enzymes used to prepare protoplasts from fungi (Wessels and Sietsma, 1979) and plants (Evans and Bravo, 1983). It is perhaps from this lytic lifestyle that the concept has developed of using *Trichoderma* and *Gliocladium* spp. as biocontrol agents against such soil-borne crop pathogens as *Gaeumannomyces graminis* Arx & D.L.Oliver (cereal take-all), *Pythium* spp. (damping-off), *Rhizoctonia solani* (root rot), *Sclerotinia sclerotiorum* and *Verticillium dahliae* (wilt) (see Volume 2, Section 2). Extrapolating from this position, it is noteworthy that

current interest in using such biocontrol agents is high, especially with regard to development of Integrated Pest Management (IPM), besides reducing the amounts (and cost) of fungicides. Conversely, *Trichoderma* and *Gliocladium* spp. can be pests through their attack of useful fungi, for example in their parasitism of mushrooms (Volume 2, Chapter 11).

3.3.3 Environmental parameters

There is some correlation between species distribution and environmental conditions. *Trichoderma polysporum* and *T. viride* occur in cool temperature regions, while *T. harzianum* is characteristic of warm climates. This correlates with optimal temperature requirements for each species (Danielson and Davey, 1973b). In general, *Trichoderma* species appear to be more prevalent in acidic soils, and Gochenaur (1970) was able to correlate the occurrence of *T. viride* with acid soils from cooler regions in Peru. *Trichoderma hamatum* and *T. pseudokoningii* are more tolerant of excessive moisture conditions than are other species. However, as a group, *Trichoderma* spp. are relatively intolerant of low moisture levels, and this has been suggested as one factor that contributes to the relatively low numbers of *Trichoderma* in the drier forest litter layers (Danielson and Davey, 1973a). In general, the distinctive characters of the individual species appear in agreement with their general occurrence though it would be interesting to have data from more extreme climatic zones.

3.3.4 Antibiotics and secondary metabolites

The opinions concerning the role of antibiotics in the development of microbial populations have wavered back and forth over the last half-century. This was in part due to the difficulties in identifying antibiotics in localized microhabitats. Today antibiotics are considered an important component in colonization and as such the early studies of *Trichoderma* and *Gliocladium* on the production of gliotoxin and viridin (Weindling, 1934) are considered to be perspicacious. There has since been major concern regarding the identification of the species and whether or not the original culture produced these compounds (Webster and Lomas, 1964). Webster's group later showed that *Trichoderma* spp. produce a range of antibiotics (Dennis and Webster, 1971a, b, c). Today the list of such compounds is even longer and includes diverse active materials: glioviridin (a diketopiperazine), sesquiterpenoids, trichothecenes (trichodermin), cyclic peptides, and isocyanide-containing metabolites (trichoviridin). Besides potentially inhibiting other microorganisms, certain of these metabolites have been implicated in the impairment of growth of higher plants and also as a cause of ill-thrift of sheep through their action in inhibiting cellulolytic rumen microbes (Brewer *et al.,* 1982). *Trichoderma* strains also produce a range of volatile inhibitory compounds that may aid in their colonization of soil (Dennis and Webster, 1971b). It is now clear that Weindling merits special credit for focussing on the role of antibiotics as ecological determinants, for his studies took place well before the practical development of penicillin in 1939 and even before the definition of the word antibiotic in the 1940s.

Trichoderma and *Gliocladium* produce a variety of secondary metabolites. These include anthroquinone pigments (pachybasin-[1,8-dihydroxy-3-methyl-9,10-anthraquinone]; emodin-[1,6,8-trihydroxy-3-methyl-9,10-anthroquinone]), whose functions are unknown, and a bewildering array of other metabolites such as benzoquinones (thermophyllin), cardinanes (avocettin); dihydrocoumarins, a branched-chain polyacetylene (trichodermene) and fatty acid derivatives (methyl-2,4,6-triene-1-carboxylate) (Slater *et al.,* 1967; Taylor, 1986; Volume 1, Chapter 7 and Volume 2, Chapter 8). The effects of these compounds on colonization is not known, but in one interesting instance a volatile (unknown structure) promoted self-fertilization in *Phytophthora infestans* (Brasier, 1971).

3.3.5 Fungistasis

Fungistasis is a further element in the development in soil of *Trichoderma* and *Gliocladium,* with both negative and positive effects being recorded. Inhibition of germination due to fungistasis has been considered a survival mechanism. This may be true although both Caldwell (1958) and Davet (1979) coincidentally recorded around a 10% loss of conidial viability over roughly a two-year period. Chlamydospores and mycelium appear less sensitive to fungistasis than conidiospores (Papavizas, 1985). For example, the introduction of pelletized biomass (mycelium and chlamydospores) into soils resulted in proliferation (1000-fold via CFU analysis). There could be other reasons for this result; for instance, the increase was favored through antibiotic production by the mature mycelium.

3.3.6 Resistance to soil treatment protocols

Trichoderma species have been known for a long time to be relatively resistant to a variety of chemicals used in soil treatments including carbon disulfide, captan, formalin, allyl alcohol, methyl bromide and Semesan (2-chloro-4-hydroxymercuriphenol). Not surprisingly, *Trichoderma* and *Gliocladium* populations can explode and become dominant after soil fumigation (Munnecke, 1972). This presumably is related to their resistance to fumigants, combined with enhancement of their colonization abilities in the absence of, or the weakened status of, competitive microbes. This post-fumigation dominance can be used effectively in combination with biocontrol application of *Trichoderma*. Physical soil treatments (e.g. steam) may also allow differential propagation of *Trichoderma* and *Gliocladium,* for instance, at the expense of the more heat susceptible *Armillaria mellea* (Munnecke *et al.,* 1981).

3.3.7 Interactions with fungi and plants

The competitive interactions between *Trichoderma* and other microbes are complex, encompassing competition for nutrients, action of lytic enzymes, besides antibiosis/ /symbiosis and fungistasis. The outcome of the pair-wise competition between the zygomycete *Mucor hiemalis* and several *Trichoderma* species is illustrative (Wardle

et al., 1993). In forest litter, *T. polysporum* initially stimulated *M. hiemalis* to produce spores rather than mycelium. In contrast, in agricultural soil *M. hiemalis* out-competed *T. harzianum,* displacing it to small microhabitats, though the relative abundance of these two fungi converged at a common level after 27 days despite varied initial inoculum ratios. In this two-fungus comparison, the dominance was dependent on a range of factors associated with the ecologic niches, namely a forest and an agricultural soil.

The diversity and interaction of factors (again somewhat ill-defined) that can control development-competing fungi is illustrated in a study of the competition between green mold *(T. harzianum)* and shiitake *(Lentinus edodes)* on sawdust (Badham, 1991). The edible mushroom was favored by increasing moisture levels above 35%, by supplementing the substrate with 4% gypsum or with moderate amounts of straw, bran or urea, or by growing on weathered oak sawdust autoclaved for 2–4 hours. The consideration of these parameters may prove helpful in minimizing green mold infection of cultivated mushrooms, but it is enigmatic that *Trichoderma,* though endowed with bounteous polysaccharases, was not favored by the addition of straw, oak or bran. The factors controlling the competition between *Trichoderma* spp. and other microorganisms are addressed more fully in Volume 2, Section 2.

However, the hyphal interactions are intriguing as dominance by *Trichoderma* could be through lysis, competition for nutrients, or the inhibitory effects of antibiotics. The action of *Trichoderma* as a mycoparasite was perhaps first recorded on *Sclerotinia* (Aytoun, 1953). As *Trichoderma* spp. are well known to produce lytic enzymes, and penetration and lysis of *Phycomyces* and *Rhizoctonia solani* (Durrell, 1968) by *T. viride* was considered of common occurrence, a strong case was made for enzymatic attack as the basis of antagonism. However, the interaction between *Trichoderma* and other fungi is not always lytic in nature. Dennis and Webster (1971c) noted that *Trichoderma* rarely penetrated *Heterbasidion annosus* cells but that *Trichoderma* hyphae simply coiled around the basidiomycete's hyphae with resultant marked inhibition of growth of the latter. "Coiling" is a classical response of *Trichoderma* towards other fungi, but the mechanism of inhibition has yet to be defined. The biochemistry of the interaction is beginning to be understood with the recent discovery of lectins produced by mycoparasitic species (Neethling and Nevalainen, 1996).

The interactions between *Trichoderma/Gliocladium* spp. and plants are intriguing and are detailed elsewhere in this volume. In brief, they can be beneficial to the plant causing growth stimulation (Kleifeld and Chet, 1992; Ousley *et al.,* 1994) or through biocontrol of plant diseases (Askew and Laing, 1994; Dewan and Sivasithamparam, 1988; Lo *et al.,* 1996; Yang *et al.,* 1995; Zhang *et al.,* 1996). In rare cases, *Trichoderma* may also be harmful or even pathogenic, to some plants under some circumstances, e.g. with some germinating seedlings (Menzies, 1993; Volume 2, Chapter 9).

The *Trichoderma/Gliocladium*-plant interaction is most complex with the presence of obligately symbiotic endomycorrhizal fungi (Calvet *et al.,* 1993; Camprubí *et al.,* 1995; McAllister *et al.,* 1994; Siddiqui and Mahmood, 1996) or other rhizosphere organisms. It should be noted that it requires major skill to correctly identify to species the obligately symbiotic endomycorrhizal fungi, of which there are more than 200 (Morton *et al.,* 1993). The tripartite plant/*Trichoderma*/endomycorrhizal system is thus more difficult to characterize than even implied by its additional

variable member. However, this tripartite system may well hold the key to greater understanding of fungal-fungal interactions (cf. Volume 2, Chapters 6 and 11).

3.4 Summary

Trichoderma and *Gliocladium* are remarkable soil fungi with the capability of utilizing diverse substrates, rapid growth, and resistance to noxious chemicals. They are dominant members of the soil fungal community, as envisaged by results from soil plating. They are generally considered as secondary decomposers of rotting biomass including being necrophytes. They include strains that are parasitic on other fungi, and here they impinge on human activities as they have become a major problem in the mushroom industry. They can also produce untoward effects through the action of their secondary metabolites, for instance, in producing ill-thrift of sheep. Conversely, it is clear that they rarely attack plants and indeed they often promote plant growth. Overall with their general role as decomposers in the soil they are beneficial to the total ecosystem and presumably aid in promoting soil fertility. Even so the conceptual ecological status of *Trichoderma* is that it is an example of an “opportunistic decomposing” soil fungus. It survives in part by its ability to produce large numbers of propagules which spend most of their lives as quiescent propagules and that commence growth upon addition of a suitable energy source. From the broad occurrence of these molds, this lifestyle is extremely effective.

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4

Sporulation and light-induced development in *Trichoderma*

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4.1 Introduction

Light has profound effects on many fungi by influencing diverse life processes, such as growth, metabolism, differentiation, pigmentation, etc. The effect of light on fungi has been the subject of considerable interest and exhaustive discussions in numerous reviews and book chapters (Betina, 1984, 1995; Carlile, 1965; Cole, 1986; Griffin, 1994; Hawker, 1966; Horwitz, 1989; Leach, 1971; Tan, 1978; Webster, 1980).

In the deuteromycete of the genus *Trichoderma,* sporulation is inducible in two different ways: starvation and/or light. When *T. viride* colonies growing in the dark induced by starvation, diffuse dark-green sporulation areas arise beginning in the older central parts of the colonies where nutrients have been partly exhausted. On the other hand, sporulation is inducible by pulses of light given to colonies growing in the dark prior to illumination. After about 24 to 30 h on photoinduced colonies, dark-green conidiation rings emerge at what have been the growth perimeters at the time of light pulses.

In this chapter, we shall discuss some known physiological, biochemical and genetic aspects of light-induced development in *Trichoderma viride*.

4.2 Physiological aspects of photoconidiation

The following physiological aspects of photoconidiation in *Trichoderma* have been mainly studied: (i) the effective wavelengths of the light used (i.e. the action spectra), (ii) the dependence of induction on the light dose, and (iii) the influence of the time intervals between individual illuminations.

4.2.1 Action spectra

T. viride reacts to exposure to the near-ultraviolet band of radiation (320–380 nm) and to blue light (380–500 nm). Björnsson (1959) observed two efficiency maxima

at 380 and 440 nm, whereas radiation above 525 nm did not induce conidiation. Kumagai and Oda (1969) found two action maxima in the near-UV region (at 320 and 380 nm) and two maxima in the visible blue portion (at 430 and 480 nm) of the light spectrum. Therefore, photoinduced conidiation in *Trichoderma* falls into the category of "blue-light effects" in fungi.

4.2.2 Illumination dose

The dependence of photoinduction of conidiation on the light dose (i.e. absorbed energy) was demonstrated in the experiments where dark-grown mycelia of *T. viride* were illuminated for various time periods with visible and/or near-UV light at 366 nm (Figure 4.1). Under the experimental conditions used, the conidiation increased for exposures up to 3 min. by daylight (intensity 550 lx). No further increase in conidiation was observed when the illumination was extended up to 30 min. With 366 nm light, up to 30 sec. of irradiation stimulated conidiation but prolonged exposure had deleterious effects (Betina and Spišiaková, 1976). The reason for the decrease in conidiation is unknown, but it might be related to other known effects of near-UV light on fungi such as inhibition of protein synthesis (Sulkowski *et al.,* 1964).

In another series of experiments, colonies of *Trichoderma* grown in the dark were subjected to photoinduction under a series of white light-emitting tubes with an intensity of 12 klx for time intervals from 1 to 240 sec. A subsequent incubation in the dark showed that illumination for 1 and 2 sec. induced only limited and retarded conidiation (white conidia). However, an induction for 4 sec. or more was sufficient to produce dark green conidiation rings on growing colonies corresponding to their perimeters at the time of photoinduction. Similar sensitivity to white light was

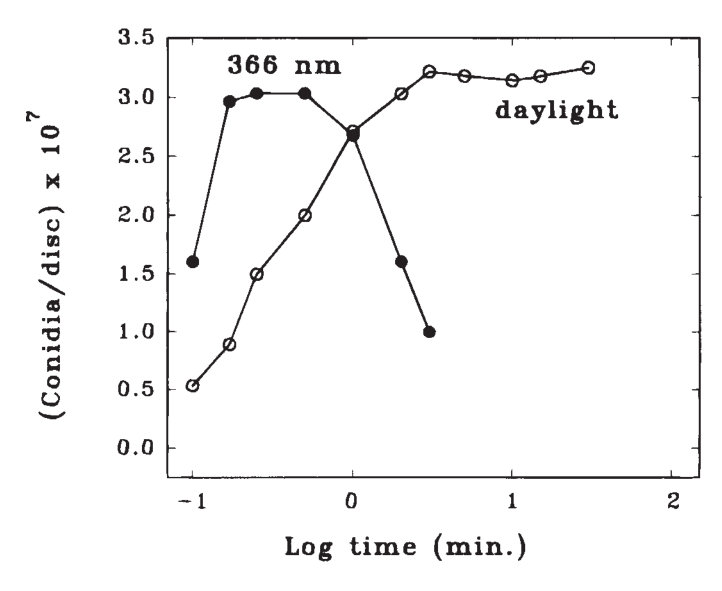


Figure 4.1 The influence of illumination time with near-UV light (366 nm) and daylight on induction of conidiation in *Trichoderma viride*. Redrawn according to data from Betina and Spišiaková (1976).

observed both in the parent strain of *Trichoderma* and in its colour mutants (V. Betina and G.B.Sharples, in preparation).

Galun (1971) induced conidiation of *Trichoderma* colonies by narrow beam illumination of restricted areas. Spot conidiation could be induced by a beam of light 0.1 mm or greater in diameter. Since the diameter of conidiated areas was greater than that of the light beam used, it appears that some kind of joint perception of photoinduction takes place among adjacent mycelial fields.

4.2.3 Is there an inherent periodicity of conidiation in Trichoderma?

Many fungi are characterized by an approximately circadian periodicity of metabolic and morphogenic processes. In *T. viride,* the production of conidiation rings depends on the frequency of irradiation and is not subject to circadian rhythm (Betina and Zajacová, 1978a).

4.2.4 Light and electron microscopic studies

It has been documented by light and electron microscopic observations (Betina, 1984; Galun, 1971; Rosen *et al.*, 1974) that the sequence of morphogenic events after photoinduction can be characterized by the steps shown in Figure 4.2: (i) outgrowth of conidiophores on which (ii) phialides are formed to give (iii) unicellular conidia that after maturation are (iv) dark-green pigmented (the changes of pigmentation during maturation are discussed in a separate section below).

The unicellular phialoconidia accumulate in slimy droplets at the tips of whorls of phialides as shown in Figure 4.3. In the case of a white conidia-producing mutant, we have also observed phialides bearing large single phialoconidia (V. Betina and G.B.Sharples, in preparation). Intracellular changes during conidium maturation, such as in mitochondria and other conidial structures, were investigated and described by Rosen *et al.* (1974).

4.3 Photoreceptor(s)

A direct consequence of the absorption of the light quantum by a sensitive molecule (photoreceptor) is the excitation of its electrons. Photooxidation is one way the

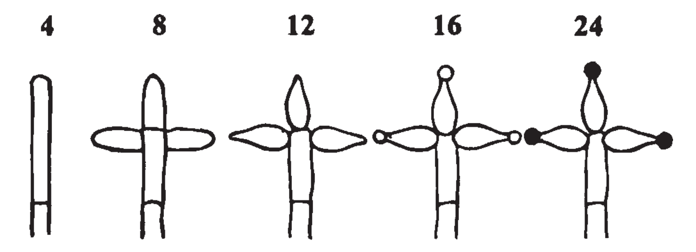


Figure 4.2 Morphological landmarks of photoinduced conidiation in *Trichoderma viride* after 4, 8, 12, 16 and 24 h from exposure to light. 4 h, outgrowth of conidiophores; 8 h, branching of conidiophores; 12 h, formation of phialides; 16 h, beginning of formation of conidia; 24 h, intensive conidia formation and their maturation. Redrawn from Betina and Zajacová (1978a,b).

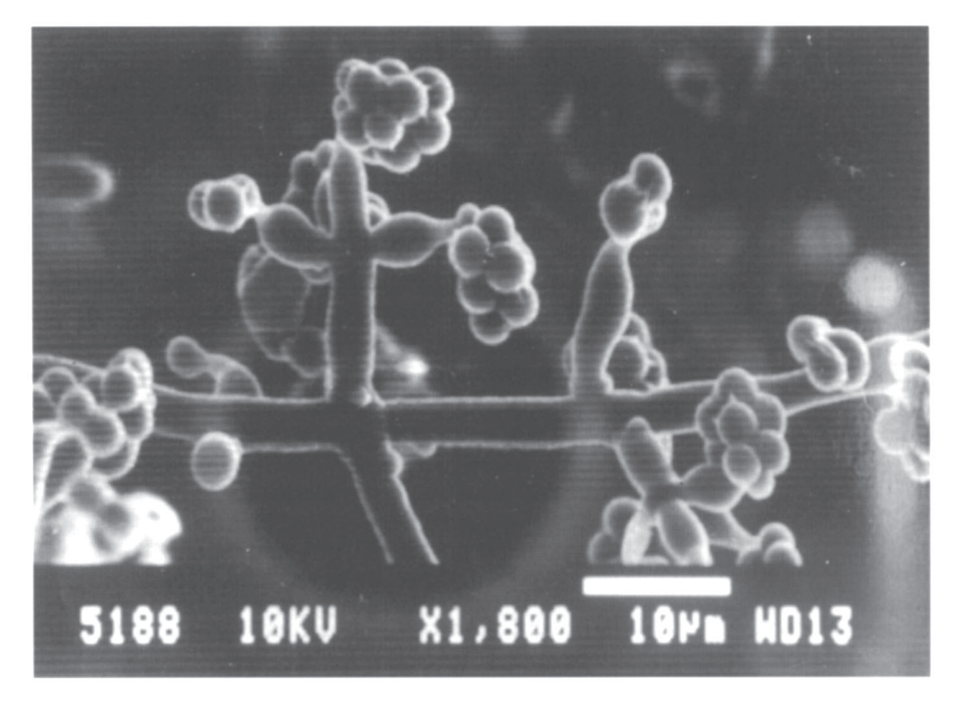


Figure 4.3 Scanning electron micrograph of *T. viride* phialides with phialoconidia. Courtesy of G.B.Sharples.

excited molecule can transfer an electron to another molecule thereby returning to ground state.

In spite of considerable efforts, the chemical identity of the photoreceptor (cryptochrome) in *Trichoderma,* and also in other photoresponsive fungi, has not yet been satisfactorily ascertained. The technique used to identify the photoreceptor has largely been based on examining the action spectra of the photoeffect while assuming that the relative intensity of the photoresponse would correspond to the relative absorbance of the photoreceptor at the given wavelenth of incoming light (Gressel and Hartmann, 1968; Kumagai and Oda, 1969). This is really an indirect approach since the resulting action spectrum may be a superimposition of spectra of different pigments, not all of them necessarily involved in the photoreception (Tan, 1978). Participation of multiple photoreceptors in the same fungus also cannot be excluded (Galland, 1983).

On the basis of resemblance of action spectra for photoconidiation to their optical absorption spectra, the main candidates for the role of photoreceptor have been flavins (flavoproteins) and/or carotenoids. Light-induced absorbance changes (LIACs) have yielded strong experimental support for the involvement of flavins in the photoreception in *Neurospora crassa* where irradiation of mycelia with blue light caused photoreduction of cytochrome b (with maximum efficiency at 465 nm), possibly mediated by a flavin (Muñoz *et al.,* 1974; Muñoz and Butler, 1975). Involvement of carotenoids was excluded by cultivating the mycelia in the dark where carotenoid production was limited. In cell-free extracts, the photoreduction of cytochromes was observed only in the presence of flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD). Indirect evidence for involvement of flavin

in the photoreception in *Trichoderma* was given by experiments where roseoflavin, a nonfunctional riboflavin analog, when added to the medium before photoinduction, inhibited blue-light induced conidiation in riboflavin requiring auxotroph Br183rib- of *Trichoderma harzianum* (Horwitz *et al.,* 1984b). In the same mutant, more riboflavin was required for blue light conidiation than for growth, whereby the extra riboflavin was required for post-photoinduction processes and not for photoinduction itself (Horwitz and Gressel, 1983).

Although available evidence speaks in favour of flavins (flavoproteins) being involved in the transduction of light signal(s), it still does not unequivocally prove that flavin is the photoreceptor itself. A possibility remains that the photoreceptor is some other, pigment and that flavin only mediates the signal by accepting the electrons from the photoexcited receptor and transferring them into the cytochrome respiratory chain (see below).

Experimental data support the notion that the photoproduct from the photoreceptor is stable and that the photoreceptor cannot be recycled. For example, increased respiration and intracellular ATP persisted only for limited time periods following the onset of illumination and their duration was independent of the duration of the light pulse. Repeated illuminations after dark periods of up to 2 hrs were practically without effect (Farkaš *et al.,* 1985; Sulová *et al.,* 1990). These results could be interpreted as indicating that there is only a limited amount of the photoreceptor in the cells and that after its pool has been exhausted (photobleached) by the initial light pulse, it has to be synthesized *de novo* before the cells are capable of a repeated photoresponse. Evidence against cycling of the photoreceptor in *Trichoderma* is supported by the finding that nanosecond pulses of light, too short to allow the cycling, were sufficient to induce conidiation (Horwitz *et al.,* 1990).

A question remains: Is the photochemical product from the photoreceptor the actual and only trigger of changes that lead to conidiation? It has been shown that pulse-illuminated colonies kept in the cold for extended periods “remember” the photoinduction and conidiate normally after transfer to optimum culture temperature (Horwitz and Gressel, 1983). This would infer that the photoproduct is stable and that it can trigger the biochemical processes leading to conidiation also after a prolonged time after photoinduction. From observations with the prokaryote *Arthrobacter,* Hoober and Phinney (1988) inferred that the photoreceptor may be a metal-chelating protein that changes its conformation and therefore its affinity toward metal ions upon illumination and, in this way, could regulate the expression of light-sensitive genes. On the other hand, the possibility to induce conidiation by starvation and the existence of *dim* (“dimsighted”) mutants of *Trichoderma,* which are unable to initiate conidiation by light but are still able to conidiate upon starvation or stress (Horwitz *et al.,* 1985a,b), suggest that multiple conidiation triggers exist.

4.4 Biochemical and physiological responses to illumination

By analogy with signalling mechanisms in other eukaryotic organisms, we may infer also in *Trichoderma* the existence of transduction pathways with involvement of elements such as photoreceptor(s), G-proteins, adenylyl cyclase, ATP, cyclic AMP (cAMP), GTP, cyclic GMP (cGMP), cAMP-phosphodiesterase, phosphoinositols, protein kinases, protein phosphatases, calcium and potassium ions, and others.

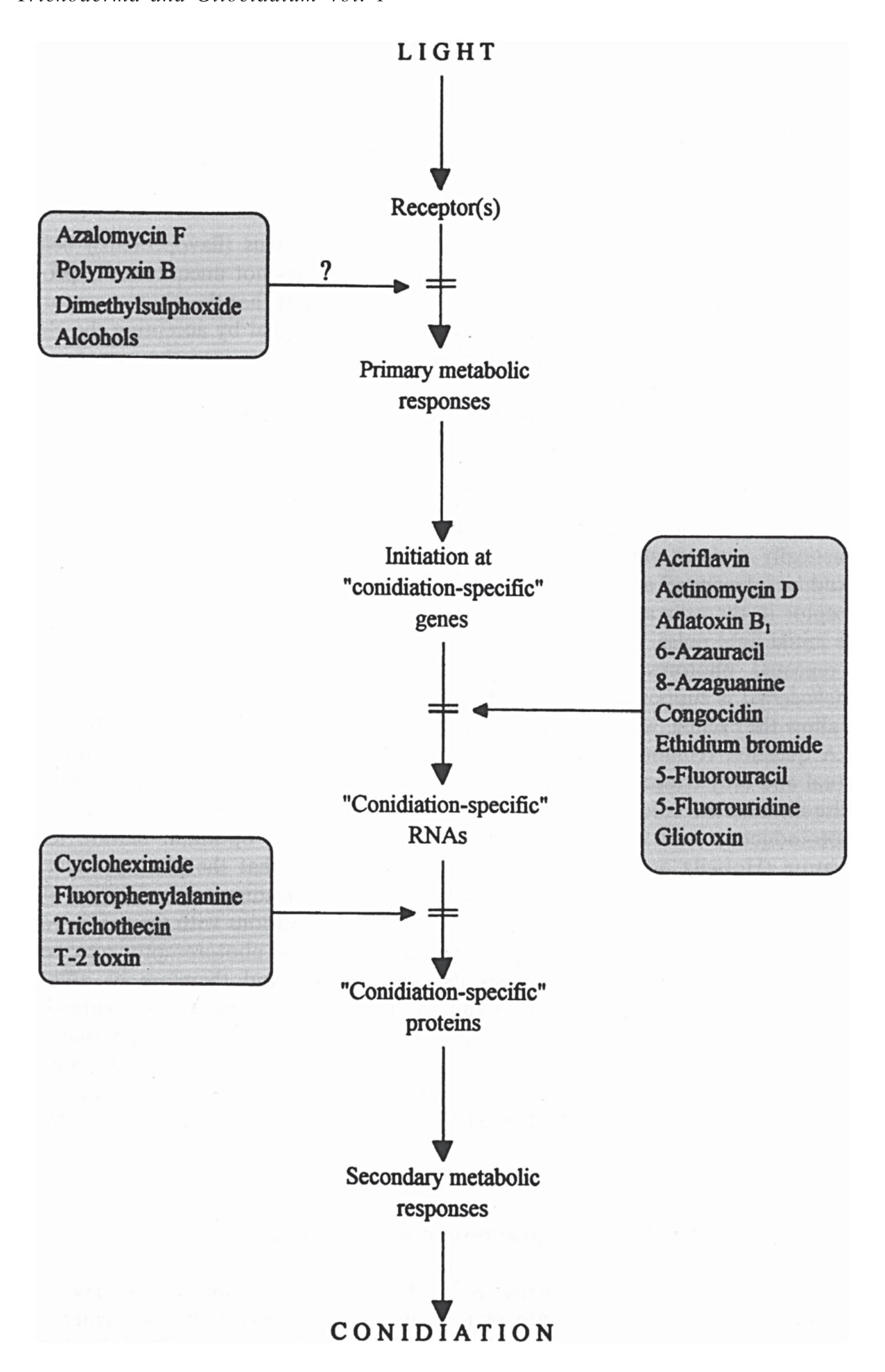


Figure 4.4 Hypothetical scheme of individual stages in photoinduced conidiation of *T. viride* and the effects of inhibitors. Reproduced with permission from Betina (1995).

In an attempt to understand the sequence of reactions taking place in the cells in the period following illumination, we shall distinguish here the primary responses, occurring within seconds to minutes, from the secondary or late responses observable only hours after the photoreception. It is highly probable that the primary responses, in contrast to secondary ones, are not dependent on changes in gene expression but are reflections of the modulation of activity of existing enzymes. The presumed order of events following the photoreception is schematically depicted in Figure 4.4.

4.4.1 Primary responses

Levels of adenine nucleotides

Among the first biochemical changes observed in dark-grown mycelia of *Trichoderma viride* exposed to white light was a rapid rise in the intracellular concentration of ATP, paralleled by an increase in the intracellular level of cyclic AMP (Figure 4.5) (Farkaš and Betina, 1977; Farkaš *et al.,* 1985; Grešík *et al.,* 1988). The rapidity of response and the relative extent of the increase in the ATP level depended on the light intensity, with measurable changes in a relatively narrow span of light intensities between 0.9 and 1.9 klx with maximum response at 1.2 klx (Támová *et al.,* 1995). Randomly selected nonconidiating mutants of *Trichoderma viride* did not respond to illumination by increased production of ATP (Farkaš *et al.,* 1985, Támová *et al.,* 1995). Examples of positive and/or negative changes in the level of intracellular cAMP following illumination have been described also in some other photosensitive fungi, e.g. *Neurospora crassa* (Potapova *et al.,* 1984; Sokolovsky and Kritsky, 1985) and *Aspergillus giganteus* (Zurzycka *et al.,* 1983), which substantiates the assumption that cAMP is involved in the transduction of the light signal. Conflicting results concerning the light-induced changes in cAMP levels have been published for *Phycomyces* (Cohen, 1974; Leutwiler and Brandt, 1983).

At present, it is difficult to ascertain whether the rise in cAMP content in the illuminated mycelia precedes the accumulation of ATP, or if both changes occur simultaneously, and whether they are independent or linked.

Examples exist in literature showing that the activity of fungal adenylyl cyclase can be stimulated by ATP (Rosenberg and Pall, 1983), but Kolarova *et al.* (1992) working with cell-free extracts from *Trichoderma viride* have found that the enzyme can directly be stimulated by light. The role of cAMP in conidiation in *Trichoderma* is further supported by observations that 1–10 mM 3-isobutyl-1-methylxantine (IBMX), an inhibitor of cAMP-phosphodiesterase, roughly doubles the formation of conidia in photoinduced mycelia of *Trichoderma,* although it was unable to induce conidiation on its own (Sulová and Farkaš, 1991). Similarly, cAMP analogs added at 1–50 μm concentrations to the growth medium stimulated conidiation in irradiated colonies and elicited the formation of conidia in *Trichoderma* colonies that were kept in the dark (Nemcovic and Farkaš, in press). It is thus very likely that the cAMP is the key element, located at the metabolic junction where different conidiation-inducing pathways meet.

Other possible secondary messengers including calcium, phosphoinositols and/ or diacylglycerol could be considered as candidates for the participation in phototransduction in fungi (Horwitz, 1989). In eukaryotic cells, intracellular Ca^{2+} influences the activity of protein-kinases and phosphatases via the Ca^{2+}-calmodulin

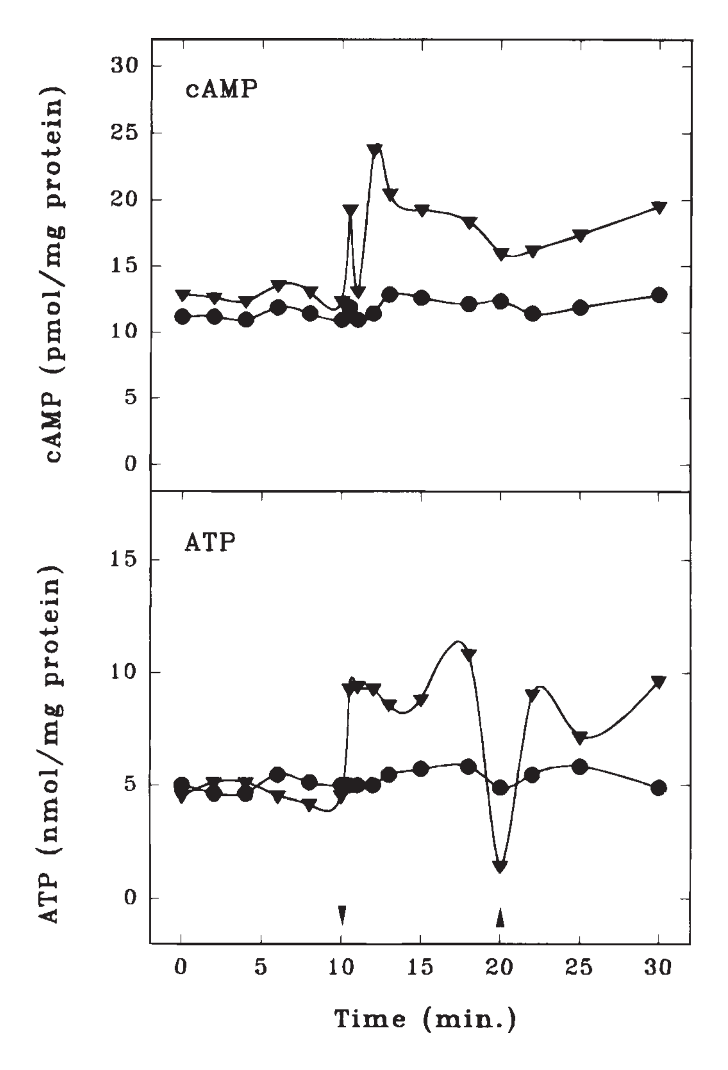


Figure 4.5 Changes in the level of intracellular cAMP (upper part) and ATP (lower part) during illumination of dark-grown mycelia of *T. viride*. The arrows indicate the period of illumination with white fluorescent lamp, intensity 1.2 klx. Redrawn according to data from Grešík *et al.* (1988).

complex. Calmodulin has been detected in some fungi, e.g. *Neurospora* (Cox *et al.,* 1982; Ortega-Perez *et al.,* 1981), where it can activate soluble adenylyl cyclase (Reig *et al.,* 1984) and cyclic-nucleotide phosphodiesterase (Ortega-Perez *et al.,* 1983). In *Trichoderma,* no clear correlation between perturbation of Ca^{2+} uptake and the inhibition of growth and conidiation were found (Kryštofová *et al.,* 1995). Non-steroidal anti-inflammatory agents stimulated both dark- and photoinduced-conidiation but inhibited growth of the fungus, indicating that arachidonate metabolism may also be important for conidiation processes (Kryštofová *et al.,* 1994).

Thus far, there is no clear evidence concerning the participation of phosphoinositols or diacylglycerol in light signalling in fungi. Preliminary experiments did not reveal any differences in composition or relative abundances of individual inositol

phosphates in illuminated and non-illuminated mycelia of *Trichoderma viride* (Kryštofová, 1996; Kryštofová *et al.,* 1998).

Respiratory burst

It is conceivable that the light-induced rise in the intracellular concentration of ATP must occur as a consequence of either activation of energy-yielding processes or by restriction of ATP consumption in the illuminated cells. A possible explanation is that the increased ATP level is an outcome of the burst of respiratory activity as indicated by oxygen uptake by dark-grown mycelia of *Trichoderma* immediately after the onset of illumination (Sulová *et al.,* 1990). Interestingly, the increase in the rate of oxygen consumption was not mirrored by increase in CO_2 formation, indicating that the stimulated respiration did not take place at the expense of increased oxidation of acetate (Figure 4.6). Inhibitors of respiration, such as antimycin A and mucidin, which block electron-transport chain (ETC) at cytochrome b/c_1 site, effectively suppressed the photoinduced respiratory burst, whereas rotenone and amytal which block transfer of electrons from NADH to coenzyme Q were without effect. Increased oxygen consumption evoked by illumination was observed also in cell-free extracts from *Neurospora* containing added FMN or FAD (Muñoz and Butler, 1975). A possible interpretation of these results is that photoexcited electrons are released from the photoreceptor and channeled into the ETC at the coenzyme Q site (Figure 4.7). Transfer of electrons along the cytochrome respiratory chain to oxygen is coupled with production of ATP at the expense of light energy, without concomitant release of CO_2. However, an alternative explanation for the increased level of ATP following illumination is indicated by the finding that isolated mitochondria from various organisms produce a sudden burst of ATP from ADP upon illumination with white light (Vekshin, 1991, and references therein).

The conidiation in *Trichoderma* is strictly an aerobic process. However, Gressel *et al.* (1975) demonstrated that the photoinduction itself can take place also in the absence of atmospheric oxygen and that the anaerobically photoinduced mycelia conidiate normally after transfer to aerobic conditions. Since in the absence of oxygen the transfer of electrons along ETC as well as the production of ATP must be limited, the importance of respiration during the photoact is uncertain.

Protein phosphorylation

Protein phosphorylation/dephosphorylation is an important regulatory mechanism, probably also involved in the light transduction in fungi (Lauter and Russo, 1990). Through increased cAMP levels, light could activate cAMP-dependent protein kinases which would phosphorylate enzymes or transcription factors involved in the regulation of light-activated genes. In experiments with membranes isolated from dark-grown mycelia, Grešík *et al.* (1989) found that light specifically stimulated phosphorylation of at least two proteins of 18 and 114 kDa. The effect of light could be substituted by 3 mM cAMP but not with 10 mM Ca^{2+}.

Electrophysiological phenomena

The transient rise in the intracellular concentration of adenine nucleotides in *Trichoderma* is accompanied by rapid hyperpolarization of the plasma membrane,

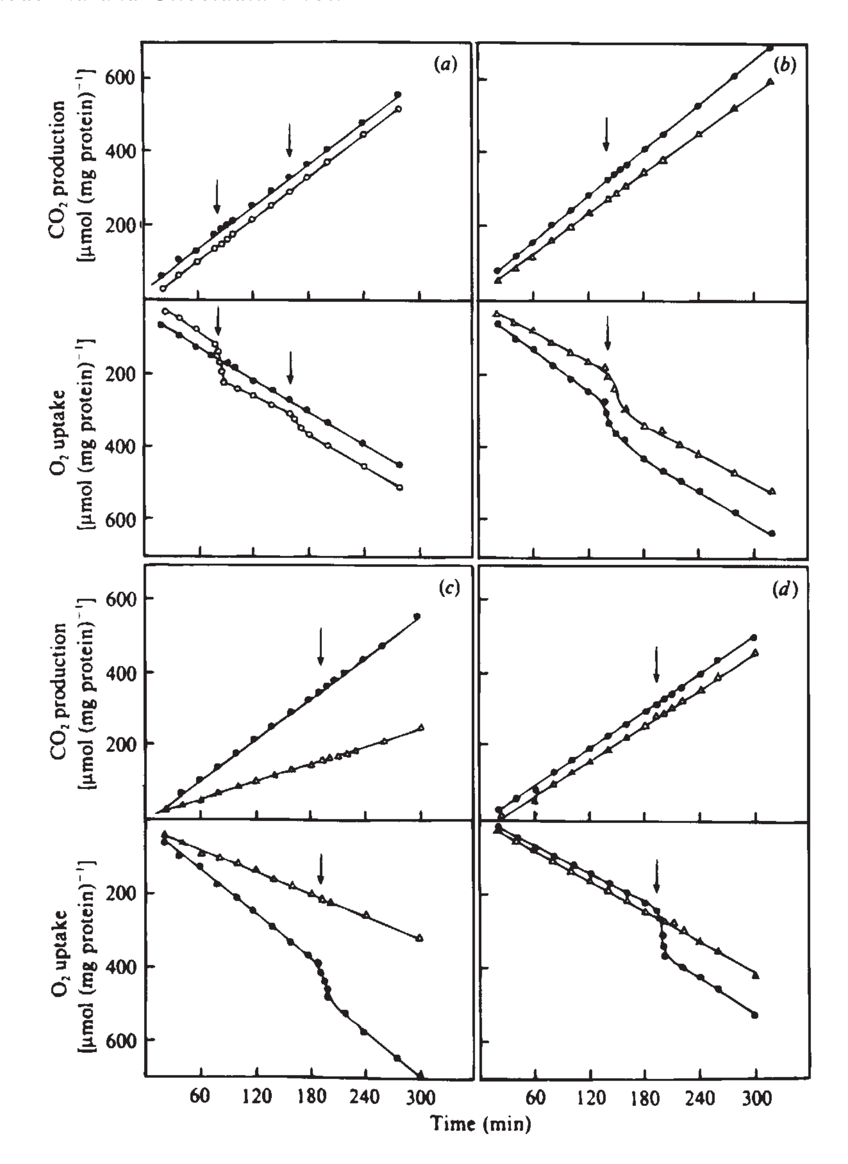


Figure 4.6 Effect of illumination on the rate of O_2 consumption and CO_2 production in mycelia of *T. viride* and the effect of inhibitors. Mycelia were grown on filter discs in the dark in the absence of inhibitors for 36 h and then transferred into manometric vessels containing agar medium with or without inhibitors. At times indicated by arrow, the mycelia were exposed to white light, intensity 1.5 klx for 10 min. (a) No inhibitors present. ○, Illuminated mycelium; ●, dark control, (b) ●, control without inhibitor; △, in the presence of 50 μM rotenone. (c) ●, control without inhibitor; △, in the presence of 20 μM antimycin A. (d) ●, control without inhibitor; △, in the presence of 1 mM dithiothreitol. Reproduced with permission from Sulová *et al.* (1990).

followed by its depolarization. At the same time, transient acidification of the cytoplasm takes place (Grešík *et al.*, 1991). It was concluded that the hyperpolarization is mainly due to light-activated efflux of K^+ ions from the cells since it could be completely suppressed by 150 mM extracellular KCl (but not NaCl or $CaCl_2$)

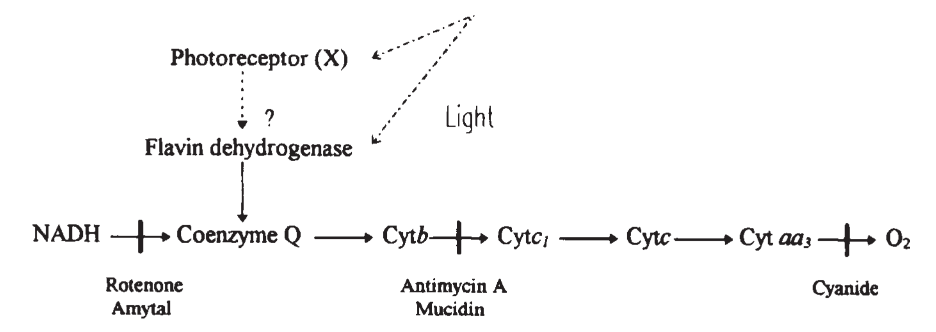


Figure 4.7 Scheme illustrating the proposed flow of electrons released by photoact from the photoreceptor along the electron-transport respiratory chain in *Trichoderma* and the inhibition sites of some inhibitors.

and/or 50 mM valinomycin. Observations made in *Neurospora* (Rodríguez-Navarro *et al.,* 1986) indicate the possibility that the intracellular acidification is due to co-transport of K^+ and H^+ ions in the phase of membrane depolarization.

An attempt to investigate the participation of extracellular electric currents in the morphological development in *Trichoderma* was made by Horwitz *et al.* (1984c). Using an external oscillating microelectrode, they detected sporadic, localized current loops at subapical regions of photoinduced hyphae, in periods from 60 to 150 min. following illumination. The significance of these currents in the process of photoinduced conidiation still has to be ascertained. They may be considered as preliminary evidence of a modification of membrane properties after photoinduction.

4.4.2 Secondary responses

The so-called secondary or late metabolic and physiological responses reflect the irreversible commitment of the organism to conidiation. Examples from fungi other than *Trichoderma* show that this could include switches in key metabolic pathways, changes in the energy metabolism, probably as the consequence of changes in expression of sporulation specific genes (e.g. Gressel and Rau, 1983, and the citations therein).

One of the late consequences of illumination in *Trichoderma* is the relative decrease in glycogen (Figure 4.8). It seems as if the cells that have been induced to conidiate were preferentially using endogenous reserves in their further development. In this context it is interesting to mention that in *Aspergillus ornatus,* light causes inhibition of glucose uptake and phosphorylation and thus, in fact, induces starvation (Hill, 1976). Such an effect would support the hypothesis that a part of the metabolic route leading from induction to conidiation is common for both the light- and starvation-induced processes (Horwitz *et al.,* 1984a).

According to the scheme in Figure 4.4, transcription of sporulation-specific genes is expected to follow the primary metabolic responses to illumination. In *Aspergillus,* there is evidence of light-induced transcription of four genes (*bli* genes) with lag times from 2 to 45 min. (Sommer *et al.,* 1989). The genes are not activated in the so-called white-collar mutants *wc-1* and *wc-2* that are insensitive to blue light.

Quantitative changes in nucleic acid and protein contents associated with photoinduced conidiation of *Trichoderma* were also reported. The contents of total

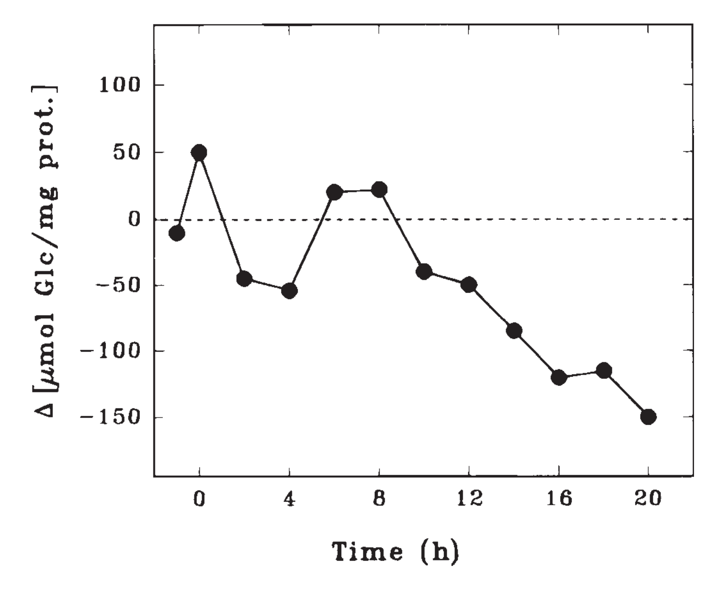


Figure 4.8 Differences in glycogen contents between illuminated and dark-control mycelia of *T. viride*. At time 0, mycelia were exposed to white light at 0.6 klx for 10 min. From Sulová (1989).

DNA, RNA and protein increased considerably after photoinduced conidiation, whereas non-induced controls exhibited only minor increases in these components. The increased contents of these macromolecular compounds after photoinduction could apparently be attributed to formation of fructification structures (Betina and Spišiaková, 1976).

Autoradiography of colonies of *Trichoderma* growing in the presence of radioactive calcium chloride showed that the radioactive Ca^{2+} was distributed homogeneously throughout the vegetative mycelium while photoinduced conidiation was accompanied by a massive accumulation of calcium in conidia (Šimkovic *et al.*, 1997).

Several changes in the appearance of the gene products following photoinduction have been observed in *Trichoderma*. For example, Horwitz and Gressel (1987) found the disappearance of a major 65 kDa protein species within 2 hr. after illumination. Glyceraldehyde-phosphate dehydrogenase is down-regulated by photoinduction at both the protein and mRNA synthesis level, whereby a protein characteristic of conidia and of conidiophores appears 12 hr. after photoinduction (Horwitz *et al.*, 1996). The transcription of the endochitinase gene *ech-42* is activated in the period following illumination suggesting, in addition to metabolic regulation, also a developmental regulation of the gene (Carsolio *et al.*, 1994). The increase in endochitinase activity may reflect the anticipated morphological changes in the illuminated mycelium. For example, an activation of breakage of chitin chains by illumination was detected at the apical regions of the cell walls of *Phycomyces blakesleeanus* sporangiophores (Herrera-Estrella and Ruiz-Herrera, 1983).

4.5 Studies with inhibitors

Various models of differentiation in eukaryotes based on DNA modifications have been proposed (Holiday and Pugh, 1975). It is conceivable that the portion of the

genome which bears information for conidiation remains somehow "silent" during the vegetative growth and becomes activated by photoreception. Therefore, it is expected that photoinduction in *Trichoderma* and other fungi will cause a "switching-on" of this silent portion of the genome. This would initiate transcription of "conidiation-specific" genes into "conidiation-specific" RNAs followed by their translation into "conidiation-specific" proteins including some enzymes. In addition, changes in the activity of some other "auxiliary" enzymes might be expected. Finally, these sequences in biochemical events should be followed by a sequence of morphological changes such as the formation of conidiophores, phialides and phialoconidia which, after maturation, become coloured dark green. Specific metabolic inhibitors were used in an attempt to reveal the role of individual metabolic pathways in photoinduction.

The first indirect evidence of transcription involvement came from Galun and Gressel (1966) and from Gressel and Galun (1967) who found that photoinduced conidiation of *Trichoderma* colonies was completely suppressed by 5-fluorouracil and 8-azaguanine, respectively, with almost no inhibition of vegetative growth. When colonies were subjected to a 5-h treatment beginning 30 min. before the induction and were then rinsed, transferred to new dishes with medium without the inhibitor, and incubated for one day, conidiation was prevented. Their results were later confirmed in our laboratory both with growing colonies and with filtered mycelium from submerged cultivation (Betina and Spišiaková, 1976). However, the contact with 5-fluorouracil from +2 to +5 h and from +3 to +6 h after photoinduction did not prevent conidiation. Hence, the delayed exposure of illuminated colonies to the inhibitor could not reverse the consequences of the photoinduction leading to conidiation. These results resemble the well-known "point of no return" in differentiation of an aquatic fungus *Blastocladiella* (Cantino, 1961). One could infer from our results that the crucial metabolic transition to conidiation that can be suppressed by inhibitors takes place within about 2 to 3 h after the photoinduction.

In another experiment, conidiation was prevented by 5-fluorouracil or cycloheximide irrespective of whether the inhibitors were present from -1 to +24 h or from -0.5 to 2.5 h with respect to photoinduction. Actinomycin D blocked conidiation on long-term treatment only (Betina and Spišiaková, 1976).

Effects of four inhibitors of RNA synthesis on growth and photoinduced conidiation of *Trichoderma* colonies were also compared (Betina and Zajacová, 1978b). While ethidium bromide, 5-fluorouracil, 8-azaguanine and acriflavine prevented conidiation to various degrees related to the concentrations used, further vegetative growth of colonies was not inhibited but rather slightly stimulated. Hence, the process of conidiation was more sensitive to these inhibitors than was vegetative growth.

The application of specific inhibitors pointed out the importance of respiration, oxidative phosphorylation and cAMP in photoinduced conidiation in *Trichoderma viride* (Sulová and Farkaš, 1991).

4.6 Genetic studies

Another avenue for investigating photoinduced conidiation in *Trichoderma* is a genetic approach. Weineman-Greenshpan and Galun (1969) and Greenshpan and

Galun (1971) were perhaps the first to prepare mutants of *T. viride* either by irradiation by X-rays and UV light or by exposure to N-methyl-N'-nitro-N-nitrosoguanidine. Non-conidiating and colour mutants were obtained and used in complementation studies by means of heterokaryosis. Most non-conidiating mutants could complement one or more of the other non-conidiating mutants. Heterokaryons of colour mutants showed both autonomous (i.e. wild-type), and/or non-autonomous (i.e. corresponding to their own genotype) character of conidial pigmentation.

In our laboratory, non-conidiating and colour mutants of *T. viride* have been prepared using UV irradiation (Bojnanská *et al.,* 1978). The colour mutants produced white, yellow or brown conidia. These mutants represent steps of conidial maturation from white through yellow and brown to green conidia.

In complementation experiments, colonies of pairs of colour mutants were allowed to anastomose, resulting in the formation of green conidia. Several pairs of mutants, which were unable to produce complementation of conidial pigmentation using this simple method, did produce green conidia when heterokaryons were produced by protoplast fusion on agar plates (Fargašová *et al.,* 1985). Except for a slowly growing white mutant (M-14), the growth rates of representative colour mutants on several cultivation media were comparable with the parent strain (Betina *et al.,* 1989).

In recent microscopic studies of the above UV-induced mutants, and of another white mutant resulting from spontaneous mutation of the brown mutant M-108, no great morphological differences were observed. More detailed data will be published elsewhere (Betina and Sharples, in preparation).

However, the brown mutant M-108 differed strongly from the parent strain in that upon illumination, it produced and excreted coloured secondary metabolites. Two of the metabolites were already isolated, purified and shown to be derivatives of anthraquinone, i.e. 1,3,6,8-tetrahydroxy-9,10-anthraquinone and 1-acetyl-2,4,5,7-tetrahydroxy-9,10-anthraquinone (Betina *et al.,* 1986). The former was already known to be produced by a strain of *Aspergillus versicolor* (Berger, 1980), whereas the latter is identical to a member of a series of anthraquinone derivatives isolated from invertebrates belonging to sea-lilies *(Crinoidea) Lamprometra kluzingeri* and *Heterometra savingii* (Erdman and Thompson, 1972); prior to our finding it was not known as a fungal secondary metabolite. Both anthraquinones functioned as uncouplers of oxidative phosphorylation in rat liver mitochondria (Betina and Kubela, 1987) but did not exhibit antimicrobial activity.

From the biochemical point of view, the accumulation of anthraquinone pigments by the brown mutant occurring in illuminated cultures only, concomitantly with pigmentation of photoinduced conidia, is very interesting. Are these anthraquinones precursors of the conidial green pigment whose biosynthesis is blocked at its final stage in the mutant? In the parent strain the anthraquinones in question (after occasional modification) would be incorporated into the final green pigment but the brown mutant would accumulate them. An analogous case was described by Ray and Eakin (1975) who had blocked the biosynthesis of aspergillin in *Aspergillus niger* conidia by inhibitors and observed the accumulation of a pentacyclic pigment or low M_r.

Bellinck (1975) extracted the green pigment of *T. viride* conidia with concentrated formic acid and NaOH (0.1 mol/L). After acid hydrolysis and alkaline degradation, 2-hydroxy-, 4-hydroxy-, 2,4-dihydroxy- and 3,5-dihydroxybenzoic acids were obtained

that were considered to be precursors of the native pigment. However, compounds of anthraquinone type, which could also be isolated when using the above procedures if they are precursors of the final pigment, were not mentioned.

In our parent strain of *T. viride,* no production of anthraquinones could be detected (Betina *et al.,* 1989) but pachybasin, chrysophanol and emodin were described as anthraquinones produced by some other *T. viride* strains (see Turner (1971) for references). However, 1-acetyl-2,4,5,7-tetrahydroxy-9,10-anthraquinone isolated from the brown mutant may originate by folding an octaketide chain differently to precursors of pachybasin, chrysophanol or emodin (Betina *et al.,* 1986).

Thus, the accumulation of anthraquinones in the brown mutant M-108 could also be explained by assuming that (i) they are shunt metabolites accumulating as a result of mutational disorders in the biosynthesis of oligoketides or polyketides and (ii) the mutation caused an impaired regulation of biosynthesis of anthraquinones presumably produced by the parent strain in minor amounts that cannot be detected by the analytical methods used (Betina *et al.,* 1989).

Most recently, Cehulová *et al.* (1996) observed that sodium vanadate at 0.1 mM concentration dramatically decreased the production of 1-acetyl-2,4,5,7-tetrahydroxy-9,10-anthraquinone by the brown mutant M-108 of *Trichoderma* and the amount of the excreted pigment dropped while its content in the mycelium remained unchanged. If molybdate was used under identical conditions, neither growth nor production of (a) secondary metabolite(s) were affected.

4.7 Concluding remarks

Photoinduced conidiation in fungi represents an exciting model for studying the biochemistry and genetics of morphological development and differentiation in eukaryotic cells. From what has been written on preceding pages, it follows that there are still too many pieces missing in the jigsaw puzzle representing the mechanism(s) by which the light signal is captured, amplified, modified and finally ensuing in the formation of conidia.

One of the key unresolved questions remains the identity of the photoreceptor (cryptochrome). The assumption that flavins and flavoproteins could play the central role in photoreception is strengthened by recent findings in a wide variety of organisms where flavoproteins structurally (but not functionally) related to photolyases have been identified as the photoreceptors (Hoffman *et al.,* 1997; Hsu *et al.,* 1996; Todo *et al.,* 1997). It is also possible that different photoreceptors exist in different fungi or even that there are multiple photoreceptors in one organism functioning either independently or in synergy, each activating a different transduction pathway.

Although biochemical and biophysical approaches to the study of fungal photomorphogenesis certainly have not reached their limits, modern methodology of molecular biology offers new possibilities to elucidate the molecular mechanisms underlying photomorphogenesis and differentiation in fungi. Mutants with defects in various stages of photoconidiation will be used to identify genes participating in these complex processes (e.g. see Horwitz *et al.,* 1985a,b). Successful characterizations of the genes *wc-1* and *wc-2* as the light-regulated zinc finger transcription factors playing a role in the light signal transduction in *Neurospora* were published recently (Ballario *et al.,* 1996; Linden and Macino, 1997).

Another important question concerns the common traits of conidiation induced by light and/or starvation. Mutants for the joint pathway as well as mutants for the light- or starvation-induced portions of the induction pathway will be useful in solving this problem (Horwitz *et al.,* 1984a).

It is hardly probable that *Trichoderma* has its own specific mechanism of transduction of the light signal. Therefore, homology with other organisms may help to reveal individual steps and to identify participating biochemical reactions. Examples could be found in *Phycomyces, Dictyostelium, Neurospora* or *Aspergillus,* where some partial reactions have been revealed to a greater detail than in *Trichoderma* (Cole, 1986). The candidates for the role of second messengers in photoinduction have been already discussed (Horwitz, 1989). Of potential importance for transduction of the light signal are G-proteins, adenylyl cyclase, protein kinases and phosphatases, adenine and guanine nucleotides, phosphoinositols, and Ca^{2+} and other ions (Hoober and Phinney, 1988). Since photoinduced differentiation is accompanied by morphological changes, the light responses might also involve the regulation of enzymes participating in the formation of the cell walls as already observed in *Phycomyces* (Herrera-Estrella and Ruiz-Herrera, 1983; Jan, 1974) and *Aspergillus* (Fiema, 1983).

Finally, we wish to conclude this chapter by a quotation from Niels Bohr's famous essay "Light and life" (Bohr, 1933):

"In every experiment on living organisms there must remain an uncertainty as regards the physical conditions to which they are subjected, and the idea suggests itself that the minimal freedom we most allow the organism in this respect is just large enough to permit it, so to say, to hide its ultimate secrets from us."

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5

Nutrition, cellular structure and basic metabolic pathways in *Trichoderma* and *Gliocladium*

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5.1 Introduction

Trichoderma and *Gliocladium* spp. are cosmopolitan and abundant inhabitants of soil, and their potential *Hypocrea* teleomorphs are known as lignicolous ascomycetes. They are therefore potent producers of a variety of plant polysaccharide degrading enzymes and capable of degrading a variety of xenobiotic materials. Many of them are recognized as fundamental organisms in forest soils due to their involvement in decomposition and nutrient cycling and their regulation of associated mycoflora. Selected strains of *Trichoderma* and *Gliocladium* spp. have also frequently been studied because of their formation of secondary metabolites (Volume 1, Chapter 8) and their use in biocontrol (Volume 2, Chapters 6 and 11). While the biochemical and molecular genetic knowledge has advanced considerably towards these targets, the basic biochemical pathways and cellular physiology of *Trichoderma* and *Gliocladium* have been dealt with only briefly. This chapter reviews the current knowledge on these topics.

5.2 Nutritional requirements and transport

A screening of the existing literature reveals that the genera *Trichoderma* and *Gliocladium* have a remarkable biosynthetic ability, as illustrated by the capability of the majority of species to grow on simple media. Being saprophytes, *Trichoderma* and *Gliocladium* spp. are able to grow on a variety of complex carbon and nitrogen substrates as well. In addition, many species have the ability to transform or even degrade potentially hazardous and persisting components.

5.2.1 Carbon nutrition

Trichoderma and *Gliocladium* spp. are famous for their ability to degrade a variety of polysaccharides (cellulose, hemicelluloses) and related polymers such as chitin. The enzymes involved in these processes are of considerable commercial importance

and are dealt with in Volume 2, Chapters 1–5. Although it is generally believed that all species of the two genera may be good enzyme producers, this idea has never been rigorously tested. In fact, Danielson and Davey (1973c) showed considerable variation in the ability of various *Trichoderma* spp. from soil to degrade different plant materials. Their ability to attack wood is particularly weak. *Trichoderma* spp. are often erroneously referred to as soft-rot fungi in literature, but this is clearly not the case, as attack only occurs by degrading nonstructural carbohydrates. *Trichoderma* spp. attack loblolly pine logs by destroying the ray parenchymatous cells and half-bordered pits (Hulme and Stranks, 1970). In pure cultures, several *Trichoderma* spp. were relatively ineffective in degrading dogwood leaves and loblolly pine needles (Danielson and Davey, 1973c), beechwood (Butcher, 1968), and birch and pine blocks (Bergman and Nilsson, 1971). The term saprophyte is therefore more appropriate and should be used in future. With regards to the selective degradative abilities, Kubicek *et al.* (1996), using genetically typified strains of *Trichoderma* sp. belonging to section *Longibrachiatum,* showed that various isolates of *T. longibrachiatum, T. citrinoviride* and *H. jecorina* (the teleomorphic form of *T. reesei;* cf. Volume 1, Chapter 2) were superior producers of cellulolytic enzymes than the other species belonging to the section (*T. pseudokoningii, H.* cf. *schweinitzii* and *H. schweinitzii*). They also noted that, on an average, isolates from tropical areas formed higher cellulase activities than those from temperate climates.

Manczinger and Pollner (1985) used carbon source utilization patterns to group *Trichoderma* species into clusters. According to their analysis, the following carbon sources were used by all strains investigated: D-glucose, D-galactose, D-fructose, D-mannose, cellobiose, trehalose, D-xylose, L-arabinose, D-mannitol, D-arabitol, glycerol, salicin, esculin, arbutin, glycerol-1-monoacetate, *ß*-methyl-D-glucoside, and *N*-acetyl-*ß*-D-glucosamine. Generally the best carbon sources seem to be glucose, fructose, mannose, galactose, xylose, trehalose and cellobiose (Danielson and Davey, 1973c). In contrast, *Trichoderma* spp. were generally unable to utilize α-methyl-D-xyloside, α-methyl-D-mannoside, methanol, ethanol, *n*-propanol, ethylamine, 5-ketogluconic acid, L-tartaric acid, propionic acid, butyric acid, oxalic acid, glyoxalic acid, DL-isocitric acid, adipic acid, DL-lactic acid, malonic acid, acetoin, maltitol, dextran, uracil, cytosine, cytidine, L-lysine, L-histidine, L-methionine, L-cysteine, α-DL-aminoadipic acid, *ß*-alanine, ethanolamine, various D-amino acids, benzoic acid, ferrulic acid, and anthranilic acid. A prominent example is *T. reesei (= H. jecorina)* which has no invertase activity and hence cannot utilize sucrose, a property which has been used to clone the *A. niger* invertase gene by complementation (Berges *et al.,* 1993). The utilization of some carbon sources (e.g. inulin, starch, xylan, pectin, lactose, sucrose, maltose, some polyols, sugar acids, most amino acids and some pentoses) is species dependent (Manczinger and Polner, 1985) and can be used for chemotaxonomic purposes.

Nelson *et al.* (1988) performed an extensive screening of various carbon compounds for potential effect on the biocontrol activity of *T. koningii* and *T. harzianum* against *Pythium* seed rot. The two fungi displayed a different response to the addition of different compounds, organic acids (particularly fatty acids) being most promotive to *T. koningii* whereas polysaccharides (e.g. starch, inulin and ribose) and polyols (e.g. arabitol) were beneficial to *T. harzianum.*

A reflection of the biochemical capabilities of species of *Trichoderma* and *Gliocladium* is the ability of different species to break down or transform a wide range of organic molecules, including several hazardous xenobiotics such as Arachlor, Malathion,

Table 5.1 Degradation of biotic and abiotic polymers by *Trichoderma* and *Gliocladium* spp.

Compound	Species	Reference	Comments
Humic substances	*T. viride*	Petrovic *et al.* (1993)	pH 6, 25°C, 10 days
Polyurethane	*G. roseum*	Pathirana and Seal (1984)	
		Pathirana and Seal (1984)	
Hydrocarbons	*Gliocladium* spp.	Oudot *et al.* (1987)	From fuel-contaminated soil; resins, asphaltens, polycyclic compounds not degraded
Poly-D,L-lactic acid polyurethanes	*Trichoderma* spp.	Owen *et al.* (1995)	Via tolylene-2,4-diamine
Thermally oxidized polyethylene	*Trichoderma virens*	Weiland *et al.* (1995)	

Dalapon (see Volume 1, Chapter 3), DDT, dieldrin, endosulfan, pentachloronitrobenzene and pentachlorophenol (Katayama and Matsumura, 1993; Patil *et al.,* 1970). Transformation reactions, particularly hydroxylations of, for example, L-tyrosine or aflatoxin, were also observed (Mann and Rehm, 1976; Sih *et al.,* 1969). Tables 5.1–5.3 present an overview of the components transformed by selected species. Smith (1995), studying the correlation between *Trichoderma* propagules and xenobiotic persistence at Mount Moosilauke in New Hampshire, USA, has argued that the organochlorine contaminant in forest soils may be influenced by the abundance of *Trichoderma* spp., particularly *T. harzianum* and *T. viride,* and that *Trichoderma* spp. actually strongly contribute to xenobiotic degradation in soil.

5.2.2 Nitrogen nutrition

Trichoderma spp. are able to utilize both complex as well as simple nitrogen sources for growth. When *Trichoderma* spp. are growing on carbohydrates as the carbon source, ammonium is generally superior to nitrate as a nitrogen source (Danielson and Davey, 1973c). Some strains such as *T. reesei* or *T. koningii* T-1 are also unable to utilize nitrate (Danielson and Davey, 1973c; Simmons, 1977). It should be noted that in the latter case this is due to a lack of the nitrate permease (E.M.Kubicek-Pranz, unpublished) and can thus not be used as an auxotrophic marker system for transformations (see Volume 1, Chapter 10).

Organic nitrogen sources such as peptone are often used in media to reduce the lag of growth on polymeric substrates such as cellulose (Mandels and Andreotti, 1978). Casamino acid mixtures are generally also adequate for good growth. It was noted (F.Latzko and C.P.Kubicek, unpublished), however, that peptone is used by *T. reesei* as both a nitrogen and carbon source and is used preferentially when supplied simultaneously with polysaccharides. While such a regulation would make sense to a saprophyte, it has not yet been investigated whether this phenomenon also occurs in other species. Among the amino acid constituents, the best organic nitrogen sources for *Trichoderma* are alanine, aspartic acid and glutamic acid (Danielson and Davey, 1973c; Jackson *et al.,* 1991). Apart from amino acids, *T. lignorum (= T. viride)* has been reported to be able to utilize purine bases, purine nucleosides and the corresponding nucleotides as sole nitrogen sources (Pommere *et al.,* 1972).

Table 5.2 Degradation of xenobiotics by *Trichoderma* and *Gliocladium* spp.

Compound	Species	Reference
Mexacarbate	*T. viride*	Benezet and Matsumura (1974)
Aniline-based pesticides[a]	*T. viride*	Kaufman and Blake (1973)
Pentachlorophenol	*T. virgatum*	Cserjesi and Johnson (1972)
Chloroguaiacols	*T. harzianum*	Van Leeuwen *et al.* (1996)

[a] Including: Propham, Propanil, Solan, Swep.

Table 5.3 Transformation reactions reported for *Trichoderma* and *Gliocladium* spp.

Substrate	Product or activity	Strain	Reference
Coumarin	4-OH-coumarin 7-OH-coumarin 6,7-OH-coumarin-6-glucoside	*G. roseum*	El-Sharkawy (1994)
Steroids	17β, 20β-dehydrogenase	*T. viride*	Lanisnik *et al.* (1992)
3(R)-hydroxybutyrate ester	Acetoacetate esters	*Gliocladium* spp.	Hasegawa *et al.* (1989)
N-carboxybenzoxy- N-*tert*-butyloxicarbonyl- N-formyl-L-tyrosine	corresponding L-dopa	*Gliocladium deliquescens*	Rosazza *et al.* (1974)
L-dopa	3,4-$(OH)_2$-phenylacetic acid 3,4-$(OH)_2$-phenethylalcohol		
D,L-pantoic acid	D-pantolactone	*Gliocladium* sp.	Sakamoto *et al.* (1993)
DL-menthyl succinate monoester	L-menthol ester	*Trichoderma* sp.	Ube Industries Ltd. (1981)
Cytosine nucleotide	Uracil nucleoside	*T. viride*	Aoki *et al.* (1972)

5.2.3 Other nutritional requirements

Elements other than carbon and nitrogen can be mobilized from inorganic sources, and there is no requirement for complex growth factors or vitamins by the majority of wild-type isolates of *Trichoderma* and *Gliocladium*. While the majority of species are able to use inorganic phosphate and sulfate as sources for phosphorus and sulfur, the respective permeases and assimilation pathways have not been studied. However, Grayston *et al.* (1986) reported some details of the oxidation of elemental sulfur by *T. harzianum* during growth on sucrose, straw or autoclaved soil as carbon sources.

The metal ion composition of *T. reesei* mycelia has been analyzed by Gaunt *et al.* (1984), and may be used to calculate the metal ion requirement. Metal ions such as iron are required for growth and can be scavenged from very low concentrations in the environment. The production of siderophores by six *Trichoderma* spp. (*T. hamatum, T. harzianum, T. viride, T. koningii, T. longibrachiatum* and *T. pseudokoningii*) was investigated by Anke *et al.* (1991). Under conditions of iron deficiency, the culture filtrate of all strains contained coprogen, coprogen B and ferricrocin. In addition, *T. longibrachiatum* and *T. pseudokoningii* produced siderophores of the fusigen type. Other metal ions are taken up by specific permeases.

Calcium uptake and homeostasis were investigated by Krystofova *et al.* (1995); calcium uptake was only slightly suppressed by agents that de-energized cells but was severely affected by heavy metal ions. Krystofova *et al.* (1996) showed that calcium influenced both vegetative growth as well as sporulation and that both processes apparently involved different biochemical mechanisms.

A number of other metal ions are also important for growth in very minute concentrations, whereas high concentrations inhibit growth. Addition of either Cd^{2+} or Hg^{2+} at 1–10 mM to *T. viride* resulted in growth inhibition and aberrant fungal morphology (Frank *et al.,* 1993). Babich *et al.* (1982) reported that Ni^{2+} was more toxic to the germination of spores of *Gliocladium* spp. than to mycelial growth. Yang *et al.* (1982) found that 10 ppm arsenate were toxic to *Trichoderma* spp. The removal of As and Hg by the formation of volatile compounds by methylation seems to be one pathway by which at least *Gliocladium roseum* counteracts their presence (Alexander, 1972; Cox and Alexander, 1973). However, like many other fungi, *Trichoderma* and *Gliocladium* spp. also trap metal ions in their cell walls (Krantz-Ruelcker *et al.,* 1993), a finding that has been employed—even with industrial mycelial wastes—for the industrial removal of metal ions from waste streams (Khalid *et al.,* 1993 a,b; Lechevalier and Drobot, 1981; Luef *et al.,* 1991).

5.2.4 Oxygen and carbon dioxide

Trichoderma and *Gliocladium* spp. are obligate aerobes, although isolates have been obtained from habitats with a very low oxygen partial pressure. Oxygen supply and mitochondrial activity have also been reported to be factors regulating cellulase formation by *T. reesei* (Abrahao-Neto *et al.,* 1995), as suboptimal levels seem to favour enzyme formation. This effect is also dependent on the type of

substrate used (Lejeune and Baron, 1995) and on the morphology of growth (Mukhopadhyay and Ghose, 1992).

Carbon dioxide, a final end-product of carbon oxidation, will inevitably accumulate to some extent in solid growth media, depending on the pH and temperature. Consequently, several *Trichoderma* spp. are inhibited by CO_2 in a pH-dependent manner: inhibition is strongest in neutral and slightly alkaline media (Danielson and Davey, 1973a). Hutchinson and Cowan (1972) reported an inhibitory effect of CO_2 and ethanol, produced by *T. harzianum,* on growth and sporulation of other fungi *(Aspergillus niger, Pestalotia rhododendri)* and on seedlings of *Lactuca sativa,* whereas no effects were observed on several bacteria (*Bacillus* sp., *Klebsiella* sp., *Pseudomonas* sp.), suggesting that some *Trichoderma* strains may be more tolerant to carbon dioxide accumulation than other fungi. Schinner and Concin (1982) reported that *Trichoderma* spp. can also carry out heterotrophic carbon dioxide fixation, which may aid this property. On the other hand, Desgranges and Durand (1990) reported that even low concentrations (2%) of carbon dioxide inhibited conidiation of *T. viride*. Carbon dioxide tolerance is probably a strain-specific property.

5.3 Influence of extrinsic factors on growth

5.3.1 Water activity

Members of the genera *Trichoderma* and *Gliocladium*—and other related fungal genera such as *Fusarium*—should be expected to be mesophilic to hydrophilic with respect to water activity. Jackson *et al.* (1991), investigating *T. virens, T. citrinoviride* and two species of *T. viride,* showed that the hyphal extension rates of all fungi declined with increasing water potential over the range -0.7 to -14.0 MPa. Within this range, all of these isolates were more tolerant to osmotic (i.e. NaCl, glycerol) potential than to matric (polyethyleneglycol) potential.

The different stages of fungal development usually differ in the critical values of environmental parameters. A decreased water activity was shown to promote sporulation of *T. harzianum,* which could be used in liquid culture to produce conidiospores with increased desiccation tolerance useful for biocontrol purposes (Harman *et al.,* 1991; Jin *et al.,* 1996). Conidial germination of *Trichoderma* spp. was found to be particularly sensitive towards increased osmotic pressure by Jackson *et al.* (1991), whereas Harman *et al.* (1991) did not detect such an effect with *T. harzianum.*

Water potential has also been reported to influence aroma formation by *T. viride,* particularly that of 2-heptanone (Gervais, 1990; Gervais *et al.,* 1988; Gervais and Sarrette, 1990). However, this observation has not been extended to the several other volatile compounds produced by various *Trichoderma* spp. A comprehensive list of aroma components produced by *T. aureoviride* has been published by Bruce *et al.* (1996).

5.3.2 Hydrogen ion concentration

The hydrogen ion concentration has a strong impact on fungal growth because many nutrients (e.g. sugars and amino acids) are taken up by symport with H^+. Fungi therefore generally favour media of slightly acid pH. There are few detailed studies on the effect of pH on the growth of *Trichoderma* or *Gliocladium* spp. in

their natural environment, although it is generally accepted that strains grow poorly at pH <7. Growth is usually optimal between pH 4 and 6.5, and only a few *Trichoderma* spp. seem to tolerate pH <3. Lejeune *et al.* (1995) using glucose-limited continuous culture showed that between pH 4 and 5 the specific growth rate, the maximum tip extension rate, and the branching frequency were highest, whereas the average hyphal diameter was smallest (2.1 μm vs. 2.5–2.7). Their data are in contrast to similar studies by Brown and Halsted (1975), who reported that the maximal specific growth rate of *T. reesei* under glucose limitation increased with increasing H^+ concentration, whereas the biomass yield Y remained constant at 0.4.

Conidiation did not appear to be influenced by changes in the pH between 2.2 and 7.6 (Lejeune *et al.,* 1995).

5.3.3 Temperature

Temperature dependency of growth seems to be an adaptive phenomenon in *Trichoderma,* as species originating from warmer climates have higher temperature optima (Danielson and Davey, 1973b). Species belonging to *Trichoderma* section *Longibrachiatum (T. citrinoviride, T. saturnisporum)* seem to have the highest temperature optima (38–44°C; Danielson and Davey, 1973b). *T. polysporum* and *T. viride* on the other hand, have been observed to be most abundant at rather cool temperatures (20–25°C) (Danielson and Davey, 1974b; Tronsmo and Dennis, 1978). Isolates of *T. viride* capable of growing even at 5°C have been reported (Jackson *et al.,* 1991; Whipps and Magan, 1987).

5.4 Cellular infrastructure and macromolecules

5.4.1 Cell walls

The cell surface of most fungi is composed of three contiguous interconnected matrices: an exocellular or capsular component, a wall component, and the plasmalemma. The definition of the boundaries between these three matrices has always been arbitrary. *Trichoderma* and *Gliocladium,* with their teleomorphs (*Hypocrea* spp.) belonging to the ascomycetes, would be expected to display cell wall structures of the chitin-*ß*-glucan type (Griffin, 1994). This has in fact been demonstrated (Benitez *et al.,* 1975; Messner and Kubicek, 1990; Nevalainen *et al.,* 1995). Cell walls of spores of *Trichoderma viride* contain polymers similar to those of the mycelial cell wall, such as *ß*-1,3-glucans, *ß*-1,6-glucans and protein (Benitez *et al.,* 1976a). However, chitin, while present in the mycelia, was lacking in the spores of *T. viride*. Melanin, which in other fungi appears associated with chitin, replaces this polymer in spore walls of *T. viride* and is located in the outmost layer (Benitez *et al.,* 1976a). Addition of polyoxin D at a concentration of 50–100 μg/ml inhibits spore germination of *T. viride* by 40–60% (Benitez *et al.,* 1976b), showing that chitin synthesis is essential for conidial germination. The resulting mycelium lost its rigidity, showing aberrant bulges along the hypha. The synthesis of the main cell wall polymers during regeneration of protoplasts was studied in *T. viride* (Benitez *et al.,* 1975) and *T. reesei* (Messner and Kubicek, 1990). Chitin appears to be absent from the aberrant tubes but present in the normal regenerating cell walls. Nevalainen *et al.* (1995) studied the differences

in the cell wall composition in the wild-type strain of *T. reesei* and one of its hypercellulolytic mutants. They detected a lower chitin content in the latter, along with a notable difference in the quantity of alkali-soluble protein.

Cell walls of filamentous fungi also contain small amounts of complex heteropolysaccharides, which were also found in *T. virens* (Gomez-Miranda *et al.,* 1990). The heteropolysaccharide bears some similarity to that isolated by Rath *et al.,* (1995) from both culture filtrates and cell walls of *T. reesei* and which has been reported to be responsible for the binding of *ß*-glucosidase and *ß*-xylosidase to the cell walls (Messner *et al.,* 1990). The enzyme content of cell walls of *Trichoderma,* and thereby the implications for protein secretion, are described in detail in Volume 1, Chapter 7.

Hydrophobins are small secreted proteins with interesting physico-chemical properties found only in filamentous fungi. These proteins were first found in *Schizophyllum commune* as genes abundantly expressed during formation of aerial structures (Schuren and Wessels, 1990). A gene *(hfb1)* encoding an abundantly-expressed 96-aa class II hydrophobin was isolated from *T. reesei* (Nakari-Setälä *et al.,* 1996). The protein was localized in the cell wall as well as in the extracellular medium. The role of this protein remains unknown but it is possible that it is involved in the attachment of the fungus to surfaces. A second hydrophobin-encoding gene was isolated more recently (Nakari-Setälä *et al.,* unpublished; gene bank accession number Y 11894), which was preferentially expressed on the conidial surface. Munoz *et al.* (1995) described the formation of aerial and submerged spores by *T. harzianum,* and observed the correlation of formation of a putative hydrophobin with spore hydrophobicity. The gene encoding this hydrophobin was recently also cloned *(srh1)* (Munoz *et al.,* unpublished; gene bank accession number Y 11841).

Finally, cell walls (and culture filtrates) of some mycoparasitic species of *Trichoderma* were also shown to contain galactose and *N*-acetyl-*ß*-D-galactosamine lectins (Neethling and Nevalainen, 1996), which may be involved in the recognition process during mycoparasitism (Volume 2, Chapter 7). Furthermore, Lora *et al.* (1994) described the presence in *T. harzianum* of a catabolite-repressible cell wall protein with a strong similarity to a chitinase inhibitor from *S. cerevisiae*. The role of the latter in protection against the mycolytic chitinases produced by these strains of *Trichoderma* is attractive, yet speculative, at the moment.

5.4.2 Nucleus

As eukaryotes, fungi contain typical nuclei surrounded by a perforated double membrane, hence separating the nucleolus from the cytoplasm. Fungal nuclei are generally smaller (1–5 μm in diameter) and contain less than 1 pg of DNA. Conidia frequently contain more than one nucleus; however, *Trichoderma* spp. seem to differ in the number of nuclei they contain. While several strains seem to be uninucleate (see, for example, Hammill, 1974a,b), whereas polynucleation is apparent in other species, e.g. *T. atroviride* ATCC 36042 (E.Heidenreich, PhD thesis, TU Wien, 1994). This topic is treated with respect to its importance for genetic transformation and asexual genetics in more detail in Volume 1, Chapters 10 and 11.

T. reesei was shown to form minute nuclei upon colchicine treatment because of abnormal nuclear division (Toyama and Toyama, 1991, 1995a). The average

DNA content of these "small nuclei" was 30% that of normal nuclei and thus aneuploid (Toyama and Toyama, 1995b). Such nuclei may be useful for transferring small amounts of DNA into protoplasts (Toyama and Toyama, 1995c).

Despite the many molecular genetic studies now carried out with *T. reesei,* biochemical work with isolated nuclei has not yet been reported. However, Belshaw *et al.* (1994) have investigated the nuclear matrix of *T. reesei* with the aim of identifying DNA sequences that bind to the internal nuclear matrix and are thus important for gene expression. In their study, an *Aspergillus nidulans* DNA fragment known to promote autonomous replication in yeast was shown to bind to the nuclear matrix; a 48-kDa protein was shown to be responsible for binding. Further studies in this area will undoubtedly have an impact on the improvement of genetic transformation systems for *Trichoderma* (see also Volume 2, Chapter 13).

The numbers and sizes of the chromosomes in different *Trichoderma* spp. have been studied in some species and the ranges are 4–7 chromosomes with sizes of 2.8–7.0 Mb (Fekete *et al.,* 1996; Herrera-Estrella *et al.,* 1993; Mäntylä *et al.,* 1992). On average, these data would be in accordance with an estimation of a total genome size of 30–35 Mb. This topic is dealt with in more detail in Volume 1, Chapters 9 and 11, and the inclusion of more information here is therefore avoided.

5.4.3 Cytoskeleton

Being filamentous organisms, the integrity of the cytoskeleton is a very sensitive point for the viability of fungi and hence also *Trichoderma* and *Gliocladium*. This subject has however not been treated in detail. The gene encoding actin *(act1),* a major component of the cytoskeleton, has been cloned from *T. reesei* (Matheucci *et al.,* 1995; S.Zeilinger, unpublished data) and shown to occur as a single gene. Two *ß*-tubulin genes (*tub1* and *tub2*) were cloned from *T. viride* (Goldman *et al.,* 1992b): mutation of $H_{(6)}$ into a Y in *tub2* was shown to confer resistance to the antimitotic drug methyl benzimidazole-2-yl-carbamate. *ß*-Tubulin mutations appear to have interesting physiological consequences, as a benomyl-resistant mutant of *T. harzianum* was shown to exhibit increased rhizosphere competence (Ahmad and Baker, 1988). Peterbauer *et al.* (1992) attributed this property to an impairment in hyphal polarity and a resulting increasing branching pattern in the mutant and a corresponding increased rate of cellulase secretion, which aids in the consumption of cellulose from the plant root mucilage.

5.4.4 Organelles and membranes

Excellent electron microscopical studies have been reported for *T. reesei* (Ghosh *et al.,* 1984, 1988, 1990), which revealed the presence of all eukaryotic organelles as also observed in other fungi. The structure of some of them (e.g. the Golgi apparatus) differs from that of higher *eukaryotes* (Ghosh *et al.,* 1990), but it is not yet known whether this is also paralleled by different biochemical properties. A more thorough discussion of ultrastructure of *Trichoderma* with emphasis on its role in protein secretion is given in Volume 1, Chapter 7.

The subsequent cells of a *Trichoderma* hypha are separated by septa. The surrounding cross-walls contain septal pores, which in many *Trichoderma* spp. frequently become blocked by septal pore plugs. This occurs both in hyphae as well as in conidiophores,

but its function is unknown. The individual cells in such a hypha are not biochemically equivalent: protein synthesis appears to be maximal at the hyphal tip and ribosomes are not transported along the hyphae as the tip advances (Stavy *et al.,* 1970). Interestingly, some *Trichoderma* spp. seem to contain rather high levels of the endoplasmic reticulum (Hammill, 1974a), which seems to be even enhancable by mutation in *T. reesei* (Ghosh *et al.,* 1982, 1984).

5.5 Primary metabolism

Metabolism serves two general functions: (1) the anabolic function that changes nutrients into the structural and functional components of the organism, and (2) the catabolic functions that extract chemical energy or nutrient elements such as N and S from complex nutrients to provide the energy and materials for anabolic reactions. Anabolism is dependent on catabolism not only for energy provision (ATP, NADH**,** NADPH) but also for the production of key intermediates for the biosynthesis of the functional macromolecules of hyphal structures. Most of the metabolic events that are important to functions of fungi in pure culture are similar to those detected in higher eukaryotes, which in turn suggests that these will also be the same in *Trichoderma* and *Gliocladium*. The reader is therefore referred to excellent reviews dealing with the primary metabolism of fungi (e.g. Griffin, 1994). Here, only those aspects will be covered in detail, where respective data have been obtained on *Trichoderma* or *Gliocladium* spp.

5.5.1 Carbon and energy metabolism

In accordance with other fungi and based on the assay of enzyme activities it can be concluded that species of *Trichoderma* and *Gliocladium* use both the glycolytic as well as the pentose phosphate pathway for carbohydrate breakdown. The extracellular oxidation of glucose or other monosaccharides, as is frequently observed with other fungi, has not been reported for *Trichoderma* or *Gliocladium. T. reesei* and *T. atroviride* definitely do not contain glucose oxidase (R.L.Mach and C.P. Kubicek, unpublished), but the *A. niger* glucose oxidase can be functionally expressed in *T. atroviride* (S.Jaksits and R.L.Mach, unpublished). In contrast, the presence of an ascorbate oxidase in *T. lignorum (T. viride)* and *T. hamatum* has been reported (Hatsutori *et al.,* 1994; Nakanishi, 1995).

Transport of glucose is catalyzed by an active transport system which required proton symport (C.P.Kubicek, unpublished). Interestingly, the *T. reesei* mutant RUT C-30, which is carbon catabolite derepressed because of a severe truncation in the *cre1* gene (Ilmen *et al.,* 1996; see Volume 2, Chapter 3), has a much lower glucose permease activity. It is not known whether this indicates regulation of this permease by carbon catabolite derepression or whether this is due to pleiotropism.

The early steps of sugar catabolism have been shown to be important for glucose control in *S. cerevisiae* (Gancedo and Gancedo, 1986). The presence of hexokinase and glucokinase and their possible involvement in carbon catabolite control has been investigated in *T. reesei* (Kubicek-Pranz *et al.,* 1991). The formation of enzyme activity with glucose or fructose as a substrate on different carbon sources suggests the presence of at least one hexo- and one glucokinase. In contrast, Samuels *et al.* (1994), carrying out isoenzyme electrophoresis of several *Trichoderma*

and *Hypocrea* spp., found a single hexokinase activity only. While this discrepancy remains to be clarified, neither enzyme seemed to have altered activity in the catabolite derepressed mutant strains *T. reesei* RUT C-30 and F4 or F5 (Labudova and Farkas, 1983), nor in two 2-deoxyglucose resistant mutants prepared during this study. This suggests that hexo- or glucokinases do not play a role in glucose control in *Trichoderma*. More recently, similar data and conclusions have been obtained for *Aspergillus nidulans* (Ruyter *et al.,* 1996).

Catabolism of glucose-6-phosphate in fungi is known to involve the glycolytic and the pentose phosphate pathways, and their relative distribution varies according to cellular needs. With respect to the latter, the presence of at least 2 isoenzymes of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase has been detected in *Trichoderma* spp. belonging to section *Longibrachiatum* (Samuels *et al.,* 1994), whereas Stasz *et al.* (1988) detected only a single enzyme in strains of *T. viride, T. harzianum, T. virens, T. koningii, T. hamatum* and *T. polysporum*. As the latter authors detected different isozymes in different strains, indicating that their methodology was capable of detecting isoenzyme differences, the difference between their results and those of Samuels *et al.* (1994) is most likely due to strain differences.

With respect to glycolytic enzymes, phosphofructokinase 2, an enzyme of major regulatory importance, has been purified and studied from *T. reesei* by Neto (1993). Unlike in yeast but consistent with earlier data in *A. niger* (Harmsen *et al.,* 1992), the enzyme is not regulated by cyclic-AMP-dependent phosphorylation but only by substrate availability.

Other glycolytic enzymes have been studied at the gene level because of their putative strong expression and hence potential use in the construction of expression cassettes (cf. Volume 1, Chapter 10). Glyceraldehyde-3-phosphate dehydrogenase has been purified from *T. koningii* and the gene has been cloned (Watanabe *et al.,* 1993). There are two isoenzymes that are distinguished by their different sensitivities against koningic acid produced by the same fungus ($I_{0.5}$ = 1 mM and 6.8 μM, respectively). The latter authors identified the reason for this difference in sensitivity by a change in aa residues 174 and 181, which are A and S instead of T and T. Interestingly, only a single glyceraldehyde-3-phosphate dehydrogenase-encoding gene was more recently cloned from *T. harzianum* (A.Herrera-Estrella *et al.,* unpublished). The authors observed a downregulation of the *gpd* transcript during light-induced sporulation.

The genes encoding 3-phosphoglycerate kinase from *T. reesei* and *T. viride* has been cloned by Vanhanen *et al.* (1989) and Goldman *et al.* (1992c). The 5'-sequences contain consensus binding sites for a cyclic-AMP responsive element, an activator protein 2, and the carbon catabolite represser Cre1. The *T. reesei pgk1* gene also contains a heat-shock consensus sequence. However, whereas the functionality of the former has not been tested, it has been shown (Vanhanen *et al.,* 1991) that *pgk1* does not respond to heat stress, hence making the mere demonstration of such elements questionable.

The gene encoding pyruvate kinase has been cloned from *T. reesei* (Schindler *et al.,* 1993). Its deduced protein structure has high similarity to that of *A. niger* and *A. nidulans* (De Graaff *et al.,* 1988), and hallmark sequences for activation by fructose-1,6-biphosphate have been found. The 5'-upstream sequences contain consensus sequences for the binding of the glycolytic regulator genes RAP1 and GCR1, which would be in accordance with the suggestion that the expression of glycolytic genes in *Trichoderma* occurs by pathways similar to those characterized

in detail in yeast. Interestingly, isoenzyme electrophoresis (Samuels *et al.,* 1994) detected 2–3 bands of pyruvate kinase, while Southern hybridizations only detected a single gene in *T. reesei* (Schindler *et al.,* 1993). There is some evidence for phosphorylation of pyruvate kinase, which may be responsible for the observation of two migration products.

The metabolic routes following glycolytic breakdown have not been studied in detail. A gene encoding the catalytic protein of pyruvate dehydrogenase has eventually been cloned from *T. harzianum* (M.Lorito, personal communication). The metabolic pathway of allyl alcohol degradation by *T. lignorum (= viride)* was studied by Jackson (1973) and shown to proceed via acrylate and acetate, while the latter was further metabolized via pyruvate acrylate accumulated at 50% (w/w) of the original substrate. Assimilation of C1-compounds such as methanol was studied by Sakaguchi *et al.* (1975a,b) in *G. deliquescens*. Using enzyme measurements and ^{14}C-radioactive labelling, they concluded that assimilation proceeded via the serine pathway. Tye and Willetts (1977) studied the growth of *T. lignorum* on methanol in continuous culture and reported that optimal growth was low (μ = 0.026) and occurred only at a low concentration of methanol (0.16%).

5.5.2 Nitrogen metabolism

As described in Section 5.2.2, most *Trichoderma* and *Gliocladium* spp. have the potential to utilize a large number of compounds as nitrogen sources, thus implying the presence of the respective assimilating enzymes. Hence, under conditions of growth with ammonium ions as the nitrogen sources, several strains of *Trichoderma (T. harzianum, T. atroviride, T. citrinoviride, T. viride, T. koningii, Hypocrea gelatinosa, T. virens)* maintain highly active levels of the two key enzymes of ammonia assimilation, glutamine synthetase and NADPH-glutamate dehydrogenase (Ahmad *et al.,* 1995). *T. harzianum* and *T. atroviride* contain single anionic forms of glutamine synthetase, NADPH-glutamate dehydrogenase and alanine aminotransferase and two isoenzymes of aspartate aminotransferase (Ahmad *et al.,* 1995). A tyrosine-inducible tyrosine aminotransferase has also been described from *T. viride* (Echetebu, 1982), which transferred the amino group to a-ketoglutarate.

Despite the ability of *Trichoderma* and *Gliocladium* to use a variety of nitrogen sources for growth, uptake of these has not been studied. However, the gene encoding a putative amino acid permease from *T. harzianum* has eventually been cloned (Vasseur *et al.,* 1995). With respect to the use of amino acids, the properties and conditions of biosynthesis of an arginase by a *Trichoderma* sp. were described (Khattab *et al.,* 1995).

Trichoderma spp., like most fungi, contain several L-amino acid oxidases (Smirnova and Berezov, 1989). In addition, however, several species (notably *T. viride*) seem to have several specific amino acid oxidases as well, e.g. L-leucine-a-oxidase (Smirnova and Berezov, 1987a,b), L-methionine-oxidase (Smirnova *et al.,* 1988a), L-phenylalanine-a-oxidase (Smirnova and Berezov, 1988b), and a-lysine oxidase (Hu *et al.,* 1994; Kusakabe *et al.,* 1988a,b; Laugaliene *et al.,* 1990; Lukasheva *et al.,* 1991; Smirnova and Berezov, 1987b, 1989; Weber *et al.,* 1994). The latter enzyme is a flavoprotein, which has been investigated in considerable detail because of its ability to inhibit tumour growth (Kusakabe *et al.,* 1988a,b). The physiological role of this enzyme has not yet been assessed. The enzyme was also immobilized

in a gelatin support and fixed on an oxygen sensor for online measurement of L-lysine in fermentation broths (Romette *et al.,* 1983).

The biosynthesis of amino acids has been investigated in *Trichoderma* or *Gliocladium* only to a limited extent. An exception is the gene encoding imidazoleglycerolphosphate dehydratase, an enzyme involved in the biosynthesis of histidine, which has been cloned from *T. harzianum* by genetic complementation of *S. cerevisiae* and used as a transformation marker (Goldman *et al.,* 1992a). Remarkably, however, selected strains appear to have the potential of overproducing some amino acids. Hence, L-cysteine was obtained with a yield of 72% (w/w) from DL-thiaproline by a *Trichoderma* sp. (Meiji Seika Kaisha Ltd, 1982). A *Gliocladium* sp. was reported to accumulate trans-4-hydroxy-L-proline during growth on a complex medium (Matsuoka *et al.,* 1993). Pitt and Bull (1981), carrying out a systematic study of the amino acid pools in *T. aureoviride* growing in continuous culture, reported that glutamic acid and alanine were the most abundant intracellular amino acids (60–100 and 43–135 μmoles per gram dry weight, respectively, between 0.06 and 0.20 h^{-1}). However, glycine, arginine and ornithine also accounted for a major portion of the total pool (5–9 μmoles per gram dry weight). Most other amino acids were present in concentrations of 1–3 μmoles per gram dry weight, and their concentrations were generally higher at higher growth rates.

Spores and mycelia of *Trichoderma* spp. accumulate certain nucleotides in levels up to 2–9 μmoles per gram dry weight (Pils *et al.,* 1991; Pitt and Bull, 1982). Concerning the biosynthesis of nucleotides, the pathway of uridine biosynthesis has been investigated in some genetic detail because of the convenient use of the corresponding mutants for DNA-mediated transformation (see Volume 1, Chapter 10). Mutants in uridine biosynthesis were isolated and characterized by Gruber *et al.* (1990a) and Manczinger *et al.* (1995). Hence, genes encoding the orotidine-5-phosphate decarboxylase *(pyr4 = ura5)* and the orotidine-5-phosphate pyrophosphorylase *(ura3)* have been cloned and characterized from *T. reesei* (Berges *et al.,* 1990; Gruber *et al.,* 1990b; Smith *et al.,* 1991) and *T. harzianum* (Heidenreich and Kubicek, 1994). It is interesting to note that the encoded proteins of *Trichoderma* and other pyrenomycetes, while highly homologous to those of other fungi, contain an insertion of about 100 amino acids whose function is not known.

5.5.3 Synthesis of lipids

Lipid biosynthesis by some *Trichoderma* spp. has been investigated in some detail. The lipid composition of the plasma membranes from *T. reesei* was investigated by Panda *et al.* (1987). They found that palmitic and oleic acids are the major constituents. Addition of surface active agents such as Tween 80 had only little effect on the composition of these lipids in the plasma membrane. Schreiber *et al.* (1986), however, showed that both exogenous fatty acids as well as phospholipid precursors increased the total amount of membrane material in *T. reesei.* The incorporation of exogenously added cholesterol into the cell membranes of *T. reesei* and *T. viride,* thereby causing an increase in the proportion of C_{14} and $C_{18:3}$ fatty acids and a decrease in the proportion of C_{18} and $C_{18:1}$ fatty acids, was also described by Biacs and Gruiz (1982). The same authors also reported that *T. viride* was more sensitive to saponins then *T. reesei,* and the resulting inhibition of growth could be used as a quantification of the saponine content.

Brown and colleagues focused on the possibility of using *T. reesei* for lipid production; the maximum quantity of lipid production on glucose as a carbon source was 16% of the dry weight, with linoleic acid being the predominant fatty acid (Brown and Hasan, 1988). Optimal pH was 3.2 and optimal temperature was determined as 27°C (Brown *et al.,* 1990). Further optimization of the phosphate and trace metal concentration in the nutrient medium increased the yield to 41.6% (Brown and Thornton, 1993). Serrano-Carreon *et al.* (1992) compared the lipid accumulation by *T. harzianum* and *T. viride* and obtained yields of 17 and 32% (w/v), respectively). Several lipid fractions from a *Gliocladium* sp. have been shown to display antioxidative properties (Burtseva and Donets, 1975).

The enzymes involved in lipid metabolism have not been studied. However, *G. roseum* has been reported to overproduce a glycerylphosphorylcholine phosphodiesterase (Imamura *et al.,* 1993).

5.6 Signal transduction and regulatory circuits

The expression of many genes is coordinately regulated by common physiological events such as growth on glucose (glucose repression), growth on ammonium (nitrogen repression), and starvation for any one of several amino acids. These co-ordinated regulatory systems or circuits are often complex and involve a variety of regulatory proteins. Furthermore, they depend on the function of signal transduction pathways which link the extracellular signals (nutrients, physical conditions) to the transcriptional response.

Only some of these regulatory circuits have been studied in *Trichoderma* spp., but it is reasonable to expect that most of those now known from work on other filamentous fungi, such as *Neurospora* or *Aspergillus,* will also occur in *Trichoderma.*

With regards to the regulation of gene expression by carbon catabolite repression, the *cre1* gene encoding a DNA-binding C2H2-zinc finger protein has been cloned from *T. reesei* (Ilmen *et al.,* 1996; Strauss *et al.,* 1995; Takashima *et al.,* 1996) and *T. harzianum* (Ilmen *et al.,* 1996). Its binding to a 5'-SYGGRG-3' consensus in the 5'-upstream region of various xylanase and cellulase genes from *T. reesei* and a chitinase gene from *T. harzianum* has been shown (Lorito *et al.,* 1996; Mach *et al.,* 1996; Zeilinger *et al.,* 1997). The function of this gene in the repression of formation of cellulases, xylanases and chitinases by glucose in *Trichoderma* spp. is described in more detail in Volume 2, Chapters 3 and 4. *In vitro* experiments have shown that *Cre1* must be phosphorylated to be able to bind to its target DNA, and dephosphorylation virtually prevents binding (R.L.Mach and C.P.Kubicek, unpublished). This is a significant difference from *S. cerevisiae,* whose equivalent Mig1p is inactivated by phosphorylation by the SNF1 kinase (Celenza and Carlsson, 1986). The activating protein kinase in *T. reesei* has not yet been identified, but the amino acid target sequence suggests that it is related to casein kinase II (R.L. Mach and C.P.Kubicek, unpublished).

Among the several cascades of signal transduction which are known to occur in higher eukaryotes or in yeast, there is evidence for the operation of at least three also in *Trichoderma:* (a) the protein kinase/phospholipid signalling pathway, (b) the Ca^{2+}/calmodulin signalling pathway, and (c) the cyclic AMP/protein kinase C signalling pathway.

Evidence for a protein kinase C signalling pathway comes from the cloning of the genes encoding a protein kinase C-homologue from *T. reesei* (Morawetz *et al.,* 1996) and of at least two 14-3-3 proteins in *T. harzianum* (Klemsdal *et al.,* 1996), as well as the use of inhibitors of PKC (staurosporin and bisindolylmaleimide) and phospholipase C (neomycin, LiCl) (D.Kristufek and C.P.Kubicek, unpublished; Wildman, 1991). An intriguing feature is the insensitivity of PKC to Ca^{2+} ions (Lendenfeld and Kubicek, 1997; Morawetz *et al.,* 1996), and the absence of regulation by autophosphorylation (Lendenfeld and Kubicek, 1997). Overexpression of *pkc1* in *T. reesei* leads to poor but highly branched growth (M.Mühlbauer, T. Lendenfeld and C.P.Kubicek, unpublished), which is consistent with the effect observed in yeast, and it may therefore be speculated that the roles of PKC in *Trichoderma* and yeast may be similar and primarily in regulating polarized growth. As the PKC protein of other fungi and yeasts, it contains an extended 400-aa N-terminus, whose function has been unknown so far but which also contains a domain with characteristics of protein binding aa-domains (Morawetz *et al.,* 1996). This was verified by Lendenfeld and Kubicek (1997), who could in fact show that a 110-kDa protein binds specifically to this N-terminus. The role of this protein is not yet known.

The presence of calmodulin has been detected in *T. viride* (Muthukumar and Nickerson, 1984). Using the calmodulin inhibitors trifluoperazine, chloropromin and quinacrine, as well as the calcium antagonists La^{2+}, the Ca-chelator EGTA and the Ca^{2+} ionophore A 23187, studies have convincingly shown the importance of a Ca^{2+}/calmodulin pathway for growth of *T. reesei* and light-induced sporulation (D. Kristufek and C.P.Kubicek, unpublished). Krystofova *et al.* (1995) have reported also that conidiation in *T. viride* is dependent on Ca^{2+} homeostasis. This signalling pathway appears to involve a Ca^{2+}/calmodulin-dependent protein kinase, which phosphorylates a 20-kDa protein (D.Kristufek and C.P.Kubicek, unpublished). Similar investigations in *Fusarium oxysporum* have shown that this 20-kDa protein has homology to transducin, thereby linking this pathway to that regulated by G-proteins (Hoshino *et al.,* 1992). While this still needs to be evaluated in *Trichoderma,* the presence of a G-protein in *T. harzianum* has been demonstrated by Inbar *et al.* (cited in Inbar and Chet, 1997), and the gene encoding the G_a-subunit has been cloned by Horwitz and Herrera-Estrella (personal communication). The possible role of G-proteins in mycoparasitic recognition is more fully described in Volume 2, Chapter 7.

The presence of a cyclic-AMP-dependent signalling pathway has been investigated by the use of inhibitors of phosphodiesterase (theophyllin; 3-isobutyl-1-methylxanthin) and by the addition of dibutyryl-cyclic-AMP. This pathway seems to be involved in transduction of light-induced sporulation, as well as the induction of cellulase formation, and is treated in detail in Volume 1, Chapter 4 and Volume 2, Chapter 3. A preliminary description of the adenylate cyclase from *T. viride* has been reported by Kolarova *et al.* (1992).

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6

Protein secretion and glycosylation in *Trichoderma*

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6.1 The secretory pathway of eukaryotic microorganisms

In yeast and animal cells, secretory proteins are synthesized on ribosomes bound to the endoplasmic reticulum (ER), from where they are subsequently translocated into the lumen of the ER by a signal sequence/receptor-mediated mechanism. In the ER, they undergo co- and post-translational modifications. Due to the number of processes occurring in the ER and their importance to protein secretion, this organelle has been considered as most important for determination of the "quality" and the "fate" of secreted proteins.

The current knowledge on the secretory pathway of eukaryotic microorganisms has largely been derived from the study of *sec*-mutants of *Saccharomyces cerevisiae* (Pryer *et al.,* 1992).

Results from ultrastructural investigations on *Aspergillus niger* and *Trichoderma reesei* support the assumption that the fungal secretory pathway follows the basic principles established in yeast and higher eukaryotes (Hemming, 1995). This is schematically shown in Figure 6.1 (from Peberdy, 1994). Proteins to be destined for secretion are directed to the ER by a translocation mechanism. Secretion is mediated by signal peptides; all secretory proteins of fungi contain cleavage peptide sequences near their N-terminal ends (for an overview on signal peptides in *Trichoderma* secretory proteins, see Table 6.1). The ER membranes harbour (among other proteins) the enzymes responsible for dolichol-dependent protein glycosylation and the lumen of the ER contains enzymes involved in protein folding such as protein disulfide isomerase (PDI), peptidyl prolyl isomerase (PPI) or chaperones such as BIP or their yeast equivalent (Kar2 proteins). Upon translocation into the ER lumen and simultaneous loss of the signal sequence, secretory proteins are therefore subject to post-translational modifications (e.g. O- and N-linked glycosylation, disulfide bond linkage formation and structural rearrangements (i.e. correct folding). Some of the genes encoding protein foldases) (M.Penttilä, personal communication) or enzyme activities (e.g. O-mannosylation activity; Kruszewska *et al.,* 1989) have already been demonstrated in *Trichoderma*. The *A. niger bipA* gene has been

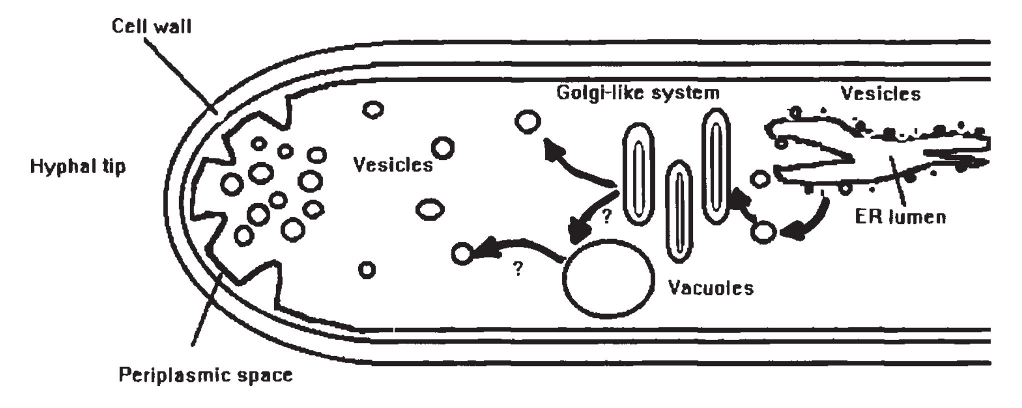


Figure 6.1 A hypothetical scheme for the secretory pathway in filamentous fungi. Proteins to be secreted are first transferred to the lumen of the ER where post-translational processes begin. Vesicles carry the molecules to the Golgi system, or its equivalent, where the processing continues. Finally, vesicles transfer the proteins to the tips of the growing hyphae where they fuse with the plasma membrane, releasing their contents into the periplasmic space. There is speculation concerning the possible involvement of vacuoles in the secretory pathway.

cloned and the work on the analysis of BipA protein has been initiated (Punt *et al.,* 1996). Genetic manipulation of the level of ER-specific molecular chaperones such as Bip-protein and catalysts for protein folding, such as protein disulfide isomerase (PDI), had increased yields of heterologously-expressed proteins in *S. cerevisiae*. Isolation of a gene, *pdiA,* coding for a putative PDI from *Aspergillus niger,* using the *S. cerevisiae PDI* gene as a probe has been recently reported by Ngiam *et al.* (1997). Genomic clone and RT-PCR products gave sequences encoding a 515 amino acid protein that included an ER-targeting translocation signal sequence of 20 aa and a mature protein of 495 aa (M_r = 54.3 kDa). The predicted protein also contained two thiol oxidoreductase active sites and a carboxy-terminal ER-retention signal (HDEL). In the *pdiA* promoter region, sequence homology to a motif associated with protein trafficking and the induction of chaperones has been identified. The same group has reported also the isolation and characterisation of a stress-inducible PDI-family gene, *tigA,* from *A. niger* and the cDNA sequence of a homologous gene, *erp38,* from *Neurospora crassa* (Jeenes *et al.,* 1997).

The transport from the ER to the Golgi involves transport via coated vesicles. Genes from *A. niger* and *T. reesei, sar1-A* and *sarT,* respectively, that encode one of the proteins involved in this process, have been reported recently (Punt *et al.,* 1996; Saloheimo *et al.,* 1996). They encode a small, GTP-binding protein. The *Trichoderma* sequence shows 72% homology compared with the yeast *SAR1* gene and 86% identity at the amino acid level with the *Aspergillus* Sar1 protein.

Further post-translational modifications occur after vesicular transfer of the proteins to the Golgi apparatus. The presence of Golgi structures in *Trichoderma* was indicated by the ultrastructural studies (Ghosh *et al.,* 1990; Kurzatkowski *et al.,* 1996) and biochemically confirmed by demonstrating of the enzymic activities typical for the Golgi in other organisms, e.g. the occurrence of Kex2 protease (Goller *et al.,* 1997), the presence of the final reactions of O-mannosylation (Kruszewska *et al.,* 1989), or the occurrence of Kex2-like proteolytic cleavage targets in prepro-aa-sequences of fungal secretory proteins. The importance of Kex2-

Table 6.1 Examples of *Trichoderma* secretory proteins; nucleotide-derived amino acid signal sequences of the leader peptides

Enzyme	Organism	Prepro-protein amino acid sequence[a]
Cellobiohydrolase I	*T. reesei*	MYRKLA*VIS*AFLATARA**Q**SA.......
Cellobiohydrolase I	*T. viride*	MYQKLA*LIS*AFLATARA**Q**SA......
Cellobiohydrolase II	*T. reesei*	MIVGILTT*LATLAT*LAASVPLEER**Q**AC......
Endoglucanase I	*T. reesei*	MAPSVTLP*LTT*AILAIARLVAA**Q**QP.......
Endoglucanase III	*T. reesei*	MNKSVAPLL*LAA*SILYGGAVA**Q**QT......
β-glucosidase I	*T. reesei*	MRYRTAAALA*LAT*GPFARADSHSTSGASAEA**V**VP
Endoxylanase II	*T. reesei*	MVSFTSLLAG*VAA*JSGVLAAPAAEVESVEKR**Q**TJ....(*)
42-kDa endochitinase	*T. harzianum*	MLSFLGKSVAL*LAA*LQATLSSASP*LAT*EERSVEKR**A**NG....(*)..
33-kDa endochitinase	*T. harzianum*	MPSLVTA*LAS*LLALVPSALAGWNVNSK**Q**NA
42-kDa endochitinase	*T. harzianum*	MLSFLGKSVAL*LAA*LQRTLSSPKPGHRRRSVEK**A**AGN
Endo-β-1,3-glucanase	*T. harzianum*	MLKLTALVALLLG*AAS*ATPJPSPPASDEGJTKR**A**TS (*)
Serine proteinase	*T. harzianum*	MTSJRRLALY*LGA*LLPAVJAA**P**A.........

[a] Putative signal sequences are indicated by italics (Watson, 1984). The underlining indicates cleavage of the KEX-like proteases. The first amino acid of the mature protein is marked in bold. Sequences marked with an asterisk contain the typical KEX 2 dibasic AA-target motif (Julius *et al.*, 1984).

1–6. Kubicek et al. *(1993); 7. Saarelain* et al. *(1993); 8. Draborg* et al. *(1996); 9. Limon* et al. *(1995); 10. Garzia* et al. *(1994); 11. de la Cruz* et al. *(1995); 12. Geremia* et al. *(1993).*

Numbers refer to the order of appearance of the names of proteins in the table.

cleavage of secretory proteins in *T. reesei* is also evident from the fact that inhibition of Kex2p results in a decreased secretion with concomitant intracellular accumulation of a non-cleaved form of the protein (Goller *et al.,* 1997). Finally, after passing the Golgi compartments, secretory vesicles will transport the proteins to the different target locations, such as the vacuole or the plasma membrane. Recent cloning of the genes encoding the GTP-binding proteins involved in vesicular transport (Saloheimo *et al.,* 1996) supports the microscopic evidence for the existence of secretory vesicles in *Trichoderma* (Kurzatkowski *et al.,* 1996). The vesicles fuse at the tips of growing hyphae with the plasma membrane, releasing their contents into the periplasmic space.

6.2 The biology of the *Trichoderma* secretory pathway

6.2.1 Ultrastructure of protein secretion in Trichoderma

Most of the earlier information on the *Trichoderma* secretory pathway has been derived from electron microscopical studies, predominantly by B.J.Ghosh and coworkers (Ghosh *et al.,* 1982, 1984, 1990; Glenn *et al.,* 1985). Earlier investigations of the ultrastructure of *T. viride* failed to reveal recognizable organelles involved in protein secretion in eukaryotes such as rough endoplasmic reticulum or Golgi apparatus. However, electron microscopical studies and cytological investigations of the *Trichoderma reesei* hypersecretory mutant RUT C-30 by Ghosh *et al.* (1984) clearly showed the presence of rough endoplasmic reticulum (rER) in *T. reesei* and its enhancement during the period of maximal cell-bound endoglucanase activity, accompanied by production of various pleomorphic bodies derived from the rER. The cells lacked, however, structures characteristic for the appearance of the Golgi apparatus. The authors discussed the possibility that the Golgi apparatus in *T. reesei* may either be morphologically uncharacteristic or that secretion may occur by a Golgi-independent pathway (Glenn *et al.,* 1985). However, the same authors later reported evidence for the first possibility (Ghosh *et al.,* 1990), which was also more recently supported by immunoelectron microscopical investigation of the structural localization of xylanase II in *T. reesei* (Kurzatkowski *et al.,* 1996).

6.2.2 Biochemical information on the Trichoderma *secretory pathway*

Further information on the secretory pathway of *Trichoderma* may be acquired from the amino acid sequences, deduced from the nucleotide sequence of the genes coding for the secreted proteins (Table 6.1). These are synthesised as precursors and contain an NH_2-terminal signal peptide that precedes the mature protein. The structure of the signal peptide exhibits homology to the consensus sequence for the ER signal peptidase (Gierasch, 1989). Most also contain the cleavage sites for the Kex2-type protease (Table 6.1).

A Kex2-like activity has recently been identified in *T. reesei* (Goller *et al.,* 1997): the enzyme is specific for dibasic and acid/basic residues, specifically inhibited by pAPMSF (p-aminophenylmethylsulfonylfluoride), and was shown to occur at a low buoyant density upon subcellular fractionation. Addition of pAPMSF to *T. reesei* cultures secreting cellulases led to an immediate halt of secretion and

the concomitant accumulation of a correspondingly larger intracellular precursor form. This indicates that preprotein processing by Kex2 is essential for protein secretion by *Trichoderma*. These properties are very similar to the one of Golgi-located Kex2-type protease from yeast (Redding *et al.,* 1991), therefore it is reasonable to assume that pre-protein processing in *Trichoderma* also takes place in the Golgi. The existence of a Kex2-type protease in *A. niger* was deduced from the cleavage of Kex2-like targets in protein fusions (Broekhuijsen *et al.,* 1993).

Another observation relevant to the *Trichoderma* secretory pathway is that recombinant bovine chymoyosin is secreted in its active form (Harkki *et al.,* 1990). Since the activation involves proteolytic trimming of prochymosin at low pH, this implies that the secretory pathway apparently involves passage through a compartment containing the appropriate protease at the appropriate pH. Based on the immunological determination that secreted proteins are present in vacuoles (see above), it is tempting to speculate that the fungal vacuole is involved in protein secretion. Such a speculation would be favoured by the detection of mannose-6-phosphate in cellobiohydrolase I (Maras *et al.,* 1997). However, clear evidence for this is lacking.

6.2.3 Physiological and mutational modulation of the efficiency of the **Trichoderma** *secretory pathway*

Secretory mutants

The isolation of *S. cerevisiae sec* genes was a major breakthrough in the understanding of the yeast secretory pathway (Pryer *et al.,* 1992). Yeast *sec* mutants, which are thermosensitive for growth, accumulate intracellular precursors of the secretory enzymes at the non-permissive temperature (Novick and Schekman, 1979). These mutants have been a starting point for the isolation of genes involved in the *S. cerevisiae* secretory pathway. There have been few attempts, however, to isolate similar mutants from *Trichoderma* or other filamentous fungi. The reason for this lies in the isolation procedure of *sec* mutants, which is based on the unicellular nature of yeast (cf. Byers, 1981) and which is not directly applicable to multicellular organisms. This is best illustrated in the attempts of Suh *et al.,* (1986) to isolate temperature-sensitive secretory mutants of *T. reesei*. Among the series of *ts* mutants isolated two categories emerged: (a) mutants that were thermosensitive for growth at 37°C and (b) mutants that were able to grow normally at 37°C if germinated at 25°C. The strains from group (b) secreted only very little protein at 37°C. However, unlike in the yeast sec mutants, no direct link between thermosensitive growth and impairment of protein secretion can be deduced.

Hypersecretory strains of *T. reesei* have eventually been obtained by selection for strains forming cellulases on cellobiose in the presence of 2-deoxyglucose (e.g. RUT C-30 and RL-P37 (Eveleigh and Montenecourt, 1979; Mandels *et al.,* 1976; Merivouri *et al.,* 1987). Although this selection strategy was primarily aimed at the isolation of mutants in carbon catabolite repression (which has most recently been proven on the genetic level by Ilmen *et al.,* 1996; see Volume 2, Chapter 4), biochemical analysis of the mutant strains revealed also several other changes including a striking proliferation of the cellular content of rER and rER-derived vesicles (Ghosh *et al.,* 1982, 1984). Furthermore, the subcellular fractionation of *T. reesei* RUT C-30 revealed

the presence of several enzymes in the proliferated ER (Glenn *et al.,* 1985), which are usually found in the Golgi bodies in other eukaryotes. Therefore, *T. reesei* RUT C-30 is an ER-mutant strain. It is not known whether this is functionally related to the mutation in the carbon catabolite represser gene *cre1* (Ilmen *et al.,* 1996), the only mutation so far characterized in this mutant.

Effect of membrane affecting agents and membrane component precursors on the Trichoderma *secretory pathway*

Addition of detergents such as Tween 80, fatty acids or phospholipids has been shown to stimulate enzyme formation in several filamentous fungi (Reese and Maguire, 1969, 1971). The biochemical basis of this effect appears to be a stimulation of limiting steps within the secretory pathway; supplementation of the growth medium of *T. reesei* with either choline or Tween 80 was shown to promote both membrane proliferation and protein secretion (Schreiber *et al.,* 1986). Further, electron microscopic examination and analysis of chemical constituents as well as marker enzymes from choline-grown mycelia revealed an increased content of mitochondria and endoplasmic reticulum when compared to the control. Therefore, the stimulation of protein secretion by choline may also be related to the relief of a bottleneck in cellular structures needed for efficient secretion and thereby resemble the effect of the RUT C-30 mutation. Similar findings were obtained for the stimulation of protein secretion by fatty acids (Panda *et al.,* 1987).

As the fusion of vesicles plays an important role at various steps of the secretory pathway, membrane fluidity is also a factor of likely importance in protein secretion. In favour of this hypothesis, the effect of several agents that affect membrane integrity or fluidity has been studied. Merivouri *et al.* (1987) showed that 2% (v/v) ethanol inhibits cellulase secretion by the hypersecretory RL-P37 strain of *Trichoderma reesei* but not by the parental strain. Interestingly, the glycosylation pattern of the secreted proteins was also altered by ethanol addition. On the other hand, Haab *et al.* (1993) reported that protein secretion by *T. reesei* RUT C-30 was considerably more tolerant to ethanol than secretion by the parent strain. This difference in ethanol sensitivity was observed with both glycosylated (cellobiohydrolase I and II) as well as non-glycosylated (xylanase II) proteins, and hence ethanol does not affect a step involved in glycosylation. Northern analysis showed that ethanol also decreased the pools of cellulase mRNA and thus interferes with the transcription of the cellulase genes. These authors explained the higher ethanol tolerance by an altered plasma membrane sterol composition, which may have resulted by selection on a medium containing bile acids during isolation of mutants.

Effect of temperature on Trichoderma *protein secretion*

The cultivation temperature has been shown to be an important and strain-specific parameter to achieve high cellulase secretion. *T. reesei* RUT C-30 forms three times as much cellulase as the parent strain at 28°C but only twice as much at 30°C (Merivouri *et al.,* 1990). B.Gassner (unpublished data) used ^{14}C-pulse labelling to study total protein secretion and showed that this temperature dependency of cellulase secretion is due to different temperature optima in *T. reesei* RUT C-30 and QM 9414, respectively. Interestingly, the different rates of protein secretion by these two *Trichoderma* strains correlated with the proportion of phosphatidylcholine

and of unsaturated fatty acids in the total phospholipid fraction, and higher proportions of both correlated with higher secretion rates (B.Gassner, D.Haab and C.P.Kubicek, unpublished data). These findings may link the effect of temperature to that of the membrane precursors as described in Section 6.4.1 but warrant further studies for a more detailed understanding.

In contrast, Merivouri *et al.* (1990) used enzyme activity assays and electrophoresis and found that an increase in temperature reduced cellulase but increased xylanase secretion, and the effect was similar for both the parental *T. reesei* QM 9414 and the mutant RUT C-30. The rate of secretion of endoglucanase by *T. reesei* QM 9414 was elevated at 17°C and comparable with the secretion of RUT C-30 at 28°C. Suh *et al.,* (1986, 1988) compared the secretion of xylanase and endoglucanase by *T. reesei* QM6a and *T. reesei* RL-P37 at 25, 30 and 37°C. Protein secretion in the low producing strain was reduced at the higher temperature but enhanced in RL-P37. Both strains, however, exhibited an increased specific activity of xylanase at the higher temperature.

6.2.4 The role of the cell wall in protein association and release

Following their secretion, many extracellular proteins from fungi remain bound to the cell wall during active growth. This has been shown for several *Trichoderma* secretory proteins; Kubicek (1981, 1982, 1983) used *ß*-glucosidase as a model to study the association of *Trichoderma* secretory proteins with its cell walls. Up to 80% of the total secreted activity of the enzyme is associated with its cell walls (Kubicek, 1981), which limits the amount of this enzyme in commercial cellulase preparations. The degree of cell wall binding of *ß*-glucosidase appears to be dependent on the cell wall composition and the dynamics of cell wall turnover. It can be released from the cell wall by incubation with *ß*-1,3-glucanase or chitinase, and an increased portion of total *ß*-glucosidase was found in the culture supernatant when *Trichoderma* cell walls contained high levels of *ß*-1,3-glucanase (Kubicek, 1982). Furthermore, release of *ß*-glucosidase into the culture fluid was enhanced during growth in the presence of the paramorphogen sorbose (Kubicek, 1983; Nanda *et al.,* 1986), a compound known to inhibit the biosynthesis of *ß*-1,3-glucan. All these data are consistent with the assumption that the *ß*-1,3-glucan fraction of the cell wall is responsible for the binding of *ß*-glucosidase to the cell wall. However, the purification and characterization of a cell wall polysaccharide that remained tightly associated with *ß*-glucosidase during enzymatic cell wall hydrolysis revealed the association with a complex heteropolysaccharide composed of mannose, galactose, glucose and glucuronic acid (Messner *et al.,* 1990). Interestingly, this polysaccharide was found both to bind *ß*-glucosidase to the cell wall as well as to activate *ß*-glucosidase *in vitro*. The structural backbone of this heteropolysaccharide, which was also shown to be responsible for the activation of *ß*-glucosidase, was determined by NMR to be a linear α-1,6-D-mannan (Rath *et al.,* 1995).

6.3 Carbohydrate structure of *Trichoderma reesei* glycoproteins

All cellulases investigated so far contain high mannose-type oligosaccharide structures attached through N-glycosidic linkages. The cellobiohydrolases and endoglucanases also contain O-linked, as well as N-linked oligosaccharides. Data have been obtained

either from chemical studies or more recently from amino acid sequence analysis. They are summarized in Table 6.2, indicating the carbohydrate content and number of potential N-glycosylation sites of the *T. reesei* cellulases.

Several separation techniques were used to analyze the products of specific glycosidase digestion and chemical degradation procedures of the glycans released from CBHI protein. It was found that CBHI contains both N- and O-linked mannans. The O-glycosidic glycans consist of one to four hexoses, presumably all mannose residues (Salovuori *et al.,* 1987). The structures of N-glycans on similar proteins differ from strain to strain. While $Man_5GlcNAc_2$ and $Man_9GlcNAc_2$ are recognized as the predominant oligosaccharides on *T. reesei* VTT-D 80133 CBHI (Salovuori *et al.,* 1987), $Man_{(7\text{-}8)}GlcNAc_2$ oligosaccharides "decorated" with phosphate and/ or glucose substituents are characterized as the predominant N-linked glycans on *T. reesei* RUTC 30 CBH I (Maras *et al.,* 1997). In general, it is accepted that *Trichoderma* N-glycans are small, compared to bakers' yeast N-glycans, and that their structures resemble those of mammalian high-mannose oligosaccharides. Similar N-glycans are present on different *Aspergillus* glycoproteins (Limongi *et al.,* 1995; Nakao *et al.,* 1987; Yamaguchi *et al.,* 1971). However, the few analysed N-linked oligosaccharides on *Aspergillus* glycoproteins show that we have to be careful with generalizations concerning fungal glycan structures. With *A. saitoi,* for instance, $Man_{(10\text{-}11)}GlcNAc_2$ on carboxypeptidase is suggested to be synthesized by mannosyltransferase(s), which are typical fungal glycosyltransferases (Chiba *et al.,* 1992, 1993). A new $Man_6GlcNAc_2$ not similar to mammalian forms was characterized on *ß*-galactosidase from *A. saitoi* (Nakao *et al.,* 1987), as was non-mammalian $Man_{(6\text{-}7)}GlcNAc_2$ on glucose oxidase from *A. niger* (Takegawa *et al.,* 1991). The $Man_6GlcNAc_2$ glycan, with α-1,6-mannose linked to Man-B of the $Man_5GlcNAc_2$ core, and $Man_{(6\text{-}7)}GlcNAc_2$, with α-1,3-mannose linked to Man-4 or Man-α of the same core, illustrate how fungal high-mannose oligosaccharides are similar in their sizes and compositions to mammalian N-glycans but not in their primary structures (Figure 6.2). In addition to mannose, galactose residues have been detected on both O-glycans and N-glycans from *A. nidulans* invertase (Chen *et al.,* 1996), *A. niger* α-glucosidase and α-galactosidase (Takayanagi *et al.,* 1992, 1994), *A. oryzae ß*-galactosidase (Nakao *et al.,* 1987) or *A. awamori* glucoamylase (Neustroev *et*

Table 6.2 Carbohydrate content of *Trichoderma* cellulases

Protein	M_r [a]	Carbohydrate content (%)	Potential N-glycosylation sites	Reference
CBHI	66 000	7	4	Shoemaker *et al.* (1983)
CBH II	47 192	11–18.6	3	Teeri *et al.* (1987)
EG I	46 036	8	6	Penttila *et al.* (1986)
EG III	42 200	15	1	Saloheimo *et al.* (1988)
EG V	22 799	—	1	Saloheimo *et al.* (1994)
BGL 1	75 341	—	7[b]	Barnett *et al.* (1991)

[a] M_r calculated from the nucleotide sequence of the respective gene.
[b] BGL 1 (β-glucosidase 1) contains 7 potential glycosylation sites, however numbers 1, 5 and 7 have proline within the Asn-X-Ser/Thr-X-Ser consensus signal that is thought to reduce the likelihood of glycosylation.

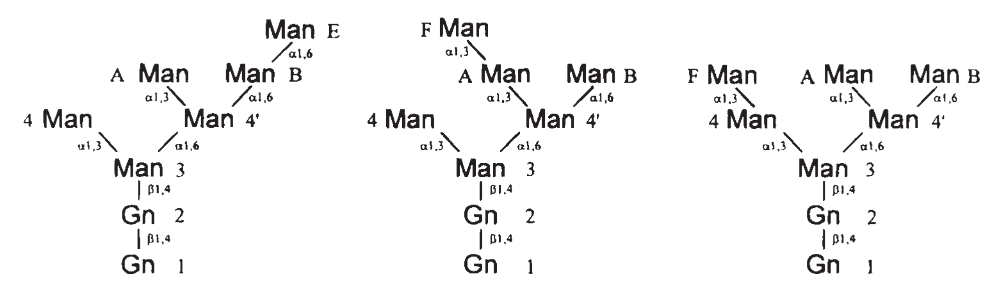


Figure 6.2 Proposed N-linked carbohydrate structures on β-galactosidase from *A. oryzae* (left) (Nakao *et al.,* 1987) and glucose oxidase from *A. niger* (middle and right) (Takegawa *et al.,* 1991).

al., 1993). The galactoses were always found to be α-linked substituents. Galactose is also present as a minor sugar component of *T. reesei* CBH I oligosaccharides, but it is not clear whether it is present in O- or N-linked glycans. Finally, mono- as well as diesterified phosphate groups were detected on *T. reesei* CBHI, their abundance varying with the strain used (Maras *et al.,* unpublished results). The importance of N- and O-glycans to thermal and/or proteolytic stability and for efficient secretion or for the biological activity is different for every fungal glycoprotein studied. It is generally accepted that oligosaccharides are determining factors for the conformation of a glycoprotein. When glycosyl structures are absent or altered, a new conformation is often formed, leading to a glycoprotein with new properties. For instance, modified O-glycans were shown to affect secretion, stability or even biological activity of *Aspergillus* glucoamylase (Goto *et al.,* 1995). An example of the importance of N-glycans for biological activity and efficient secretion is that of *Rhizomucor pusillus* aspartic proteinase. It was demonstrated that a complete inhibition of N-glycosylation resulted in intracellular accumulation of the glycoprotein (Murakami *et al.,* 1993). In contrast, N-glycans did not seem to be essential for secretion of *T. reesei* endoglucanases I and II (Kubicek *et al.,* 1987).

Another interesting feature of the cellulase structure is the occurrence of a highly O-glycosylated region typically rich in serine and threonine. This region occurs once at the C-terminus of CBHI and EGI and even in duplication in the amino terminus of CBHII. The function of these O-glycosylated domains is described in more detail in Volume 2, Chapter 1. Kubicek *et al.,* (1987), using low concentrations of 2-deoxyglucose to inhibit cellulase O-glycosylation, observed a decrease in the secretion of endoglucanases as well, suggesting that O-mannosylation of proteins may be essential for protein secretion.

6.4 Dolichol-dependent protein mannosylation in *Trichoderma reesei*

In view of the significant occurrence of O-mannosylation in *T. reesei* cellulases, its biosynthesis was studied in detail by Kruszewska *et al.* (1989). Fungal O-mannosylation is unique, as it involves dolichylphosphate (PD) and is initiated already at the level of the endoplasmic reticulum (Herscovics and Orlean, 1993; Tanner and Lehle, 1987). The sequence of reactions as well as the enzymes involved are shown in Figure 6.3; mannosylphosphodolichol (MPD) is formed in a reaction (catalyzed by MPD-synthase) where the mannose residue is transferred from

Dol-P + GDP-Man	⇒	**Dol-P-Man + GDP**
Dol-P-Man + Protein-(Ser/Thr)	⇒	**Protein-(Ser/Thr)-Man + Dol-P**
Protein-(Ser/Thr)-Man + GDP-Man	⇒	**Protein-(Ser/Thr)-(Man)** $_{4\text{-}5}$**. + GDP**

Figure 6.3 *In vitro* biosynthesis of O-linked oligosaccharides in *Trichoderma reesei*. The first mannosyl residue is donated by DolPMan; subsequent mannosyl residues are transferred from GDPMan.

GDPMan to the lipid acceptor (PD). The first mannose donated to a serine or threonine residue is transferred with MPD as a donor *in vitro,* whereby its configuration is inversed (Tanner and Lehle, 1987). More recently, Orlean (1990) provided genetic evidence for the involvement of MPD in the mannosylation of *S. cerevisiae* glycoproteins. The PMT1 gene, which encodes MPD::protein O-mannosyltransferase, has been cloned (Strahl-Bolsinger *et al.,* 1993). Gene disruption led to a complete loss of *in vitro* mannosyltransferase activity, using MPD as donor and a peptide as acceptor, in the enzymic activity assay. *In vivo,* it was observed, however, that protein O-mannosylation in the disruptant had decreased only to about 40– 50%. Hence, the authors postulated the existence of (an) additional transferase(s), which were not detectable by the *in vitro* enzyme assay. In fact, the existence of the *PMT2* gene, coding for a protein which shows significant similarity with the MPD::protein O-mannosyltransferase has been reported more recently by Lussier *et al.* (1995). Yeasts carrying a *PMT2* disruption show diminished *in vitro* and *in vivo* O-mannosylation activity and resemble mutants with a nonfunctional *PMT1* gene. Strains bearing a *pmt1/pmt2* double disruption show a severe growth defect but retain residual O-mannosylation activity. Hence, the authors postulate the presence of at least one more Pmt-protein. The second and subsequent mannose residues are transferred directly from GDPMan (Tanner and Lehle, 1987). Based on the results of O-mannosylation in a *S. cerevisiae SEC18* mutant, which is blocked in the transfer between ER and the Golgi, it was concluded that the first and second mannose residues are added in the ER (Kuranda and Robbins, 1991; Tanner and Lehle, 1987). The latter hypothesis has to be reconsidered, however, since there is no evidence that GDPMan can be transported into the ER lumen. Further elongation of the O-linked glycan chains occurs in the Golgi (Herscovics and Orlean, 1993).

With *T. reesei,* O-mannosylation of proteins has been demonstrated *in vitro* (Kruszewska *et al.,* 1989): a 40000 × g membrane fraction of *T. reesei* QM 9414 catalysed mannosyl transfer from [^{14}C]-GDPMan to endogenous lipids and proteins. Both reactions were strongly dependent upon the addition of exogenous dolichylphosphate. These findings are consistent with the conclusion that a *T. reesei* QM 9414 membrane fraction was responsible for O-mannosylation of the endogenous protein acceptors. The *in vitro* kinetics of protein glycosylation indicate that formation of mannosyl-lipid occurs prior to transfer of sugar moieties to the protein. This is consistent with the assumption that mannose is transferred to the protein via a lipid intermediate, tentatively identified as mannosylphosphodolichol. Further support for this was obtained by pulse-chase experiments using cold

GDPMan and by using tsushimycin, a specific inhibitor of MPD-synthase (Elbein, 1981). The latter substance virtually blocked the incorporation of [U-^{14}C]-mannose into both lipid and protein. All of these results sustain the conclusion that the lipid intermediate is MPD and that the initial protein-O-mannosylation occurs via this lipid intermediate.

[U-^{14}C] mannose was transferred *in vitro* from MPD to endogenous membrane proteins. Approximately 90% of the [^{14}C]-mannose transferred to protein in the above *in vitro* assay was released by mild alkaline treatment (ß-elimination). TLC analysis of the released oligosaccharides showed that they consisted of mono-, tri- and tetramannosyl-O-glycans.

Kruszewska *et al.* (1989) further observed a 2.5-fold higher activity of MPD-synthase in lactose-grown *Trichoderma*. This is in agreement with the following suggestion: the rate of endogenous mannosyl transfer was higher in mycelia grown under carbon catabolite derepressed conditions (lactose as carbon source) as compared to mycelia grown under carbon catabolite repressed conditions (glucose as carbon source). As this difference was not observed when an excess of cold GDPMan was included into the assay mixture, carbon catabolite repressed mycelia apparently contain lower concentrations of MPD and/or GDPMan (Kruszewska, 1991). Quantitative analysis of polyprenols in the membranes of *T. reesei* QM 9414 revealed a very low concentration (about 5 μg per gram mycelial dry weight) (Kruszewska, unpublished) in comparison to human liver tissue (452 μg/g) (Chojnacki and Dallner, 1988).

6.4.1 Enzymes of the protein glycosylation pathway

Dolichol kinase

The occurrence of dolichol kinase (DolK), the enzyme catalysing the transfer of *?*-phosphate from CTP into dolichol, has been demonstrated in membrane fractions from *T. reesei* QM 9414 (Kruszewska *et al.,* 1994). In *S. cerevisiae,* DolK was shown to play a major role in the regulation of available lipid intermediates (Heller *et al.,* 1992) and hence of the efficiency of glycosylation. In contrast to MPD-synthase, the activity of DolK in *T. reesei* QM 9414 was not affected by the presence of glucose in the medium. Mycelia grown at elevated temperatures (35°C) contained over proportionally increased activities of DolK and other enzymes involved in protein O-mannosylation, yet the enhanced activity of MPD-synthase and protein mannosyltransferase was only apparent in the absence of exogenous dolichylphosphate in the assay (Table 6.3). This suggests that membranes from mycelia grown at elevated temperatures contain increased amounts of dolichol and dolichylphosphate. This is further supported by stimulation of DolK activity (isolated from *T. reesei* grown at 25°C) by a total lipid extract of *T. reesei* grown at 35°C. Temperature therefore appears to influence the dolichol content in the membranes.

MPD-synthase

It has been found (Kruszewska *et al.,* 1990) that stimulation of exoprotein secretion in *T. reesei* QM 9414 by choline and Tween 80 correlates with a significant increase

Table 6.3 Influence of temperature on the activity of various enzymes involved in protein O-mannosylation in *T. reesei* QM 9414

	MPD-synthase		MPD-transferase		Dol Kinase
T (°C)	DolP−	DolP+	DolP−	DolP+	Dol+
25	16	431	66	36	21
35	20	360	92	32	47

Enzyme activity is calculated as pmoles of product formed per mg of membrane protein in 5 minutes.
Abbreviations: MPD-synthase, mannosylphosphodolichol synthase; MPD-transferase, mannosylphosphodolichol: protein O-mannosyltransferase; Dol, dolichol; DolP, dolichyl phosphate; Dol Kinase, dolichol kinase.

of MPD-synthase activity. The effect was more pronounced upon cultivation of the fungus in carbon catabolite derepressed media, e.g. in the presence of lactose as compared to glucose. Interestingly, strain RUT C-30 when grown in the presence of choline or Tween 80 exhibited a decreased activity of MPD-synthase and was not affected in its protein secretion. Simultaneously, a lack of effect of either choline or Tween 80 on MPD-synthase activity *in vitro* was demonstrated. Significant differences were observed with respect to MPD-synthase activity only, whereas other ER-located glycosylating enzymes were not affected. MPD-synthase from *Trichoderma,* as with the analogous enzyme from yeast and animal cells, is stimulated by phosphatidylcholine (PC) *in vitro*. It was thus postulated that increased MPD-synthase activity might result from an enrichment of the ER-membranes in phosphatidylcholine. On the other hand, however, the measurement of the PC concentration of intracellular membranes of *T. reesei* QM 9414 and RUT C-30 did not reveal significant differences (Kubicek *et al.,* unpublished data).

Based on the described effect of choline and Tween 80 on protein secretion and MPD synthase activity, it was hypothesized that the activity of MPD-synthase might be one of the rate-limiting points in protein secretion by *T. reesei* (Kruszewska *et al.,* 1989, 1990). To obtain molecular proof of this, a recombinant strain of *T. reesei,* which harbours the *S. cerevisiae* DPM1 (MPD-synthase-encoding) gene under a constitutive promoter of *T. reesei,* was constructed. The recombinant strain exhibited moderately elevated MPD-synthase activity, a roughly ten-fold increase in secreted cellulase activities, and a six-fold increased amount of CBHI relative to the parent strain (Kruszewska *et al.,* 1997). Since integration of genes using "strong" *Trichoderma* promoters is widely used, it will be of interest to employ this technique to elucidate further the observed stimulatory effect. The result obtained, however, is difficult to interpret since in *S. cerevisiae* MPD has been demonstrated to play important roles in at least three cellular processes (Orlean, 1990): (i) provision of the mannosyl donor for sugar chain elongation of the oligosaccharide linked to dolichyl phosphate during the synthesis of N-glycosidic linkages, (ii) supply of the first O-glycosidically-linked mannose residue, and (iii) synthesis of the sugar moiety of phosphatidylinositol (PI) anchor, which anchors some proteins in the membranes. Which of the above processes (if any) is affected in *T. reesei* upon overexpression of the yeast MPD-synthase-encoding gene and its relationship to the efficacy of protein secretion are currently under investigation.

GTP: α-D-mannose-1-phosphate guanyl transferase

GDP-mannose acts as a mannose donor for the synthesis of both N- and O-linked glycans as well as lipid-glycan intermediates (e.g. DolPP-$GlcNAc_2Man_9Glc_3$). It is currently believed (for review see Abeijon and Hirshberg, 1992; Herscovics and Orlean; 1993, Tanner and Lehle, 1987) that the first five mannose residues are added to a dolichol-linked disaccharide (DolPP$GlcNAc_2$) directly from GDPMan, on the cytosolic side of endoplasmic reticulum, whereas the subsequent four Man residues are transferred from MPD on the lumenal side. In higher eukaryotic cells (e.g. retina and thyroid tissue), GDPMan acts not only as a substrate for mannosyltransferase(s) but also indirectly stimulates the biosynthesis of the non-mannose-containing GlcNac-lipids (Kean, 1980; Ronin *et al.,* 1981). An indirect stimulatory effect of GDPMan on protein O-glycosylation in *T. reesei* has been reported by Kruszewska *et al.* (1990). A cDNA fragment, coding for *T. reesei* guanyl-transferase has been isolated by Kruszewska *et al.,* (in preparation). Nucleotide sequencing of the clone revealed an ORF of 1.6-kb, putatively encoding a protein composed of 354 amino acids, which exhibited 81% and 71% identity, respectively, with the *S. cerevisiae* guanyl transferase gene (MPG1).

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Secondary metabolism in *Trichoderma* and *Gliocladium*

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7.1 Introduction

The discovery of penicillin, a secondary metabolite of *Penicillium notatum* West (= *P. chrysogenum* Thom), in 1929 marked a milestone in the development of antibiotics. In the intensive studies that followed this discovery, mycologists and microbiologists searched for further sources of antibiotics by investigating microbial antagonism and antibiosis. In one of the earliest studies, Weindling (1932) observed that a strain of what he believed to be *Trichoderma lignorum* was antagonistic to other fungi. The antibiotic agent was exuded into the cultivation medium (Weindling, 1934) and could be isolated as a crystalline compound (Weindling and Emerson, 1936). The same compound, which exhibited high fungicidal activity, was isolated from *Gliocladium fimbriatum* Gilman and Abbott (= *Myrothecium verrucaria* (Albert and Schw.) Ditmar ex Fr.) (Weindling, 1937) and the structure of this novel compound, named gliotoxin, was established almost 30 years later (Beecham *et al.,* 1966). Since these pioneering efforts, interest in the secondary metabolites produced by *Trichoderma* and *Gliocladium* species has been sustained and the isolation and characterization of a considerable number of metabolites has been achieved. Interestingly, gliotoxin and related compounds have recently attracted much interest in the medical field as potential immunomodulating agents that have the ability to suppress the immune system by triggering cell suicide (apoptosis) (Jiang *et al.,* 1993). An increasing number of metabolites from *Trichoderma* and *Gliocladium* species are being discovered by the application of bioassay-guided separation. With this technique, a biochemical assay is used to identify metabolites in an extract exhibiting a particular biochemical activity. In this way, biological activities other than antibiotic properties have been recognized for many compounds produced by *Trichoderma* and *Gliocladium* species.

The aim of this chapter is to highlight the variety of secondary metabolites isolated from species of *Trichoderma* and *Gliocladium,* to emphasise the general classes to which they belong and to outline the major secondary biosynthetic pathways available to these organisms. Moreover, this compilation is intended to

facilitate future chemical work on these species by providing a ready reference to the compounds already characterised and a biosynthetic framework within which new examples from known classes of compounds can be allocated. It is hoped that this review will provide the beginnings of a database on which arguments on chemotaxonomy may be founded.

A certain amount of confusion has surrounded the taxonomy of *Trichoderma* and *Gliocladium* and a reliable and precise system for species and strain identification is urgently required (Samson, 1995). The species involved in the production of gliotoxin (Weindling, 1934), gliotoxin and viridin (Brian and Hemming, 1945; Brian *et al.,* 1946), originally considered to belong to *Trichoderma,* were subsequently identified by Webster and Lomas (1964) as *Gliocladium* species. A report describing the isolation of viridiol from the culture filtrate of *Trichoderma viride* Pers. ex Gray contains a footnote stating that this isolate "is the Weindling G-1 strain…also described as *Gliocladium fimbriatum* Gilman and Abbott" (Moffatt *et al.,* 1969). A paper by Bu'Lock and Leigh (1975) refers to the biosynthesis of gliotoxin in "*Trichoderma viride* (probably a *Gliocladium* sp.)" and another (Golder and Watson, 1980) to the biosynthesis of viridin in *Trichoderma viride*. *Gliocladium virens* Miller *et al.*, the producer of gliotoxin and viridin in these studies, is currently considered to be *Trichoderma virens* (Miller *et al.*) von Arx (Volume 1, Chapter 1). To avoid confusion, the species names mentioned in the primary literature have been retained in this review.

7.2 Primary and secondary metabolites

The basic features of energy production and cell growth in all organisms are essentially similar and the compounds involved in the fundamental reactions of growth and reproduction are the same. These ubiquitous and essential compounds—amino acids, carbohydrates, fatty acids, nucleic acids—are classified as primary or intermediary metabolites. Not surprisingly, despite this overall fundamental unity, differences still occur between organisms. For example, the amino acid lysine is biosynthesised in plants and bacteria from aspartate and pyruvate and in fungi from oxoglutarate and acetyl CoA.

Another feature of primary metabolites is that their concentrations in cells are strictly controlled and kept very low to avoid undesirable non-enzymatic side reactions and feedback inhibition. However, regulation and coordinating processes in some cases may be defective. As a consequence, under certain conditions both plants and microorganisms can accumulate considerable quantities of primary metabolites. Citric acid, an obligatory intermediate in the TCA cycle, may accumulate in high quantities in *Aspergillus niger* at low pH and in the absence of metal ions. *Corynebacterium (= Clavibacter)* spp. can accumulate 60 g dm^{-3} of glutamic acid although the requirement for growth is only 0.3 g dm^{-3} (Haslam, 1986).

In contrast to the ubiquitous nature of primary metabolites, there is a much greater number of compounds biosynthesised by organisms that appear not to have a crucial role in the organisms that produce them. These natural products have commonly been referred to as "secondary metabolites", reflecting the inability to assign a role to them in the internal economy of the organism. In general, they are compounds of limited molecular weight (<3000 daltons) and great structural variety. The idea that these compounds serve no "essential" function initially

deterred biologists from investigating them. For a considerable time, secondary metabolites were only of interest to the organic chemists concerned primarily with the isolation, identification and synthesis of these metabolites rather than with aspects of fungal metabolism and ecological interactions.

Secondary metabolites are broadly divisible into several characteristic groupings—polyketides, terpenes, phenols, alkaloids—that reflect their origin and biosynthesis. The essential difference between secondary and primary metabolites is that for the former a wide range of compounds can arise from one single intermediate by slight variations of the metabolic pathway. Moreover, different species of the same family, and different isolates of the same species, can often produce significantly different compounds leading to the suggestion that secondary metabolites "express the individuality of species in chemical terms" (Bu'Lock, 1965). On the other hand, widely separate species can produce the same class of secondary metabolite and sometimes even the same secondary metabolite. Gibberellic acid, the plant growth hormone present in small amounts in plants, is produced in large quantities by the fungus *Gibberella fujikuroi* (Sawada) Wollenw. Gliotoxin is produced by a number of *Penicillium* species, *Aspergillus fumigatus* Fres. and *Thermoascus crustaceous* (Apinis and Chesters) Stolk. For more detailed discussions on the distinctions between primary and secondary metabolites, the interested reader is referred to the reviews of Bennet and Bentley (1989), Betina (1994), Haslam (1986), and Luckner (1990).

For the purposes of this chapter, we define secondary metabolites as those compounds that accumulate in the organism and, to a large extent, are metabolically inactive towards the organism that produces them (end-products). Strains able to make these compounds may lose, because of mutations, the capacity to synthesise them without any apparent immediate effect to the organism. We also take the view that secondary metabolites express the biochemical differentiation of the organism. The view that these compounds represent evolution in progress and improve the survival fitness of the organism will have to await a better understanding of the interactions between organisms before it can be validated.

7.3 Types of secondary metabolites

In contrast to primary metabolites, which comprise several hundreds of compounds, secondary metabolites number over one hundred thousand, and many thousands are discovered every year. The formulation of rules for their biosynthesis has brought some order to what appears to be a chaotic collection of different structures and an "untidy jungle of uncouthly named extractives" (Bu'Lock, 1961). These rules have been developed by intuitive speculations and, subsequently, have been supported and refined by studies with isotopically labelled precursors and the use of enzyme preparations. In this review, the various secondary metabolites produced by *Gliocladium* and *Trichoderma* species are presented in terms of accepted classifications (Luckner, 1990; Turner and Aldridge, 1983). Since details of the biosynthetic pathways involved are known for only a few metabolites, a certain amount of liberty has been taken in assigning some metabolites to a particular biosynthetic class. The structures of the secondary metabolites and the biosynthetic pathways involved in their formation are collected in figures and schemes. The source of each

Table 7.1 Secondary metabolites from *Trichoderma* and *Gliocladium* species

Figure 7.1		
1	(mannitol)	
	T. hamatum	Hussain *et al.* (1975)
	T. pseudokoningii	Kamal *et al.* (1971)
2	(2-hydroxymalonic acid)	
	T. pseudokoningii	Kamal *et al.* (1971)
3	(methyl benzoate)	
4	(*p*-hydroxybenzyl alcohol)	
	T. koningii	Huang *et al.* (1995a)
5	(ferulic acid)	
	G. virens	Dickinson *et al.* (1995)
6	(2,5-dimethoxybenzoquinone)	
	T. pseudokoningii	Kamal *et al.* (1971)
7	(dihydrocoenzyme Q10)	
8	(coenzyme Q10)	
	G. roseum ACC 650	Bentley and Lavate (1965)
Figure 7.2		
9	(succinic acid)	
10	(itaconic acid)	
	T. pseudokoningii	Kamal *et al.* (1971)
11	(pencolide)	
	T. album	Ren (1977)
12	(carolic acid)	
	Trichoderma sp.	Turner and Aldridge (1983)
13	(viridiofungin A)	
14	(viridiofungin B)	
15	(viridiofungin C)	
	T. viride ATCC 74084	Harris *et al.* (1993)
Figure 7.3		
16	methyl-2,4,6-octatriene carboxylate	
17	(trichodermene A)	
	T. pseudokoningii	Kamal *et al.* (1971)
Figure 7.4		
18	(2,4,6,8-nonatetrone-2,8-bis-ethyleneketal)	
	G. zaleskii	Nadkernichnaya *et al.* (1986)
19	(2,3-dihydroxy-5,6-dimethyl benzoquinone)	
	G. roseum	Pettersson (1964, 1965)
20	(2,3-dimethoxy-5,6-dimethyl benzoquinone)	
	G. roseum	Brian *et al.* (1951)
21	(2-methoxy-3-hydroxy-5,6-dimethyl benzoquinone)	
	G. roseum	Pettersson (1964, 1965)
22	(2,3-dimethoxy-5,6-dimethyl quinhydrone)	
	G. roseum	Brian *et al.* (1951)
23	(2,3-dimethyl-5,6-dimethoxy-2,3-dihydro benzoquinone)	
	G. roseum	Brian *et al.* (1951), Grove (1966)
24	(3,5-dihydroxy toluene)	
25	(1,2-dimethyl-3,4-dihydroxy benzene)	

Table 7.1 *(Cont)*

26	(2,3-dimethyl-4,6-dihydroxy benzoic acid)	
	G. roseum CMI 93065	Pettersson (1964, 1965)
27	(1-hydroxy-3-methyl anthraquinone)	
	T. viride PRL 2233	Slater *et al.* (1967)
	T. harzianum IMI 311089	Ghisalberti *et al.* (1990)
28	(1,8-dihydroxy-3-methyl anthraquinone)	
	T. viride PRL 2233	Slater *et al.* (1967)
	T. harzianum IMI 311089	Ghisalberti *et al.* (1990)
29	(1,6,8-trihydroxy-3-methyl anthraquinone)	
	T. viride PRL 2233	Slater *et al.* (1967)
30	(1,3,6,8-tetrahydroxy anthraquinone)	
31	(1,3,6,8-tetrahydroxy-4-acetyl anthraquinone)	
	T. viride	Betina *et al.* (1986)
32	(trichodermaol)	
	Trichoderma spp.	Adachi *et al.* (1983)
33	(dimeric xanthone)	
	Trichoderma sp. SC 2051	Manyu (1980)
34	(sorbicillin)	
35	(bisvertinol)	
36	(bisvertinolone)	
37	(trichodimerol)	
37a	(trichodermolide)	
37b	(sorbiquinol)	
	T. longibrach. ATCC2449	Andrade *et al.* (1996)
Scheme 7.4		
38	(nectriapyrone)	
	G. vermoesenii IMI 40231	Avent *et al.* (1992a,b)
39	(vermopyrone)	
	G. vermoesenii IMI 40231	Avent *et al.* (1992a,b)
40	(harzianopyridone)	
	T. harzianum IMI 298371	Dickinson *et al.* (1989)
Scheme 7.5		
41	(harzianolide)	
	T. harzianum IMI 311092	Almassi *et al.* (1991)
	T. harzianum IMI 298371	Claydon *et al.* (1991)
	T. harzianum	Ordentlich *et al.* (1992)
42	(dehydro harzianolide)	
	T. harzianum IMI 311092	Almassi *et al.* (1991)
43	(harzianic acid)	
	T. harzianum SY-307	Sawa *et al.* (1994)
Scheme 7.6		
44	(trichoharzin)	
	T. harzianum	Kobayashi *et al.* (1993)
44a	(compactin)	
	T. longibrachiatum	Endo *et al.* (1986)
	T. pseudokoningii	Endo *et al.* (1986)

Table 7.1 *(Cont)*

Figure 7.5	
45 (6-pentyl-α-pyrone)	
T. harzianum IMI 275950	Claydon *et al.* (1987)
T. harzianum IMI 284726	Claydon *et al.* (1987)
	Hanssen and Urbasch (1990)
T. harzianum ATCC 20672	Merlier *et al.* (1984)
T. koningii IMI 308475	Simon *et al.* (1988)
	Hanssen and Urbasch (1990)
	Cutler *et al.* (1986)
	Benoni *et al.* (1990)
Trichoderma spp.	Kikuchi *et al.* (1974)
Trichoderma spp.	Hill *et al.* (1995)
T. viride	Collins and Halim (1972)
	Hanssen and Urbasch (1990)
T. viride 0101	Prapulla *et al.* (1992)
46 (6-pent-1-enyl-α-pyrone)	
T. harzianum IMI 275950	Claydon *et al.* (1987)
T. harzianum IMI 284726	Claydon *et al.* (1987)
T. viride	Moss *et al.* (1975)
47 (massoilactone)	
Trichoderma spp.	Hill *et al.* (1995)
48 (δ-decenolactone)	
Trichoderma spp.	Hill *et al.* (1995)
49 (koninginin E)	
T. harzianum IMI 311090	Ghisalberti and Rowland (1993)
T. koningii ATCC 46314	Parker *et al.* (1995b)
50 (koninginin D)	
T. harzianum IMI 311090	Dunlop *et al.* (1989)
51 (koninginin B)	
T. harzianum IMI 311090	Almassi *et al.* (1991)
T. koningii ATCC 46314	Cutler *et al.* (1991b)
52 (hydroxy koninginin B)	
T. harzianum IMI 311090	Ghisalberti and Rowland (1993)
53 (koninginin A)	
T. harzianum IMI 311090	Almassi *et al.* (1991)
T. koningii ATCC 46314	Cutler *et al.* (1989)
54 (koninginin C)	
T. koningii ATCC 46314	Parker *et al.* (1995a)
55 (seco-koninginin)	
T. harzianum IMI 311090	Ghisalberti and Rowland (1993)
Scheme 7.9	
56 (cyclonerodiol)	
T. harzianum IMI 311090	Ghisalberti and Rowland (1993)
T. koningii ATCC 46314	Cutler *et al.* (1991a)
T. koningii	Huang *et al.* (1995a)
57 (cyclonerodiol oxide)	
T. polysporum	Fujita *et al.* (1984)
58 (epicyclonerodiol oxide)	
T. polysporum	Fujita *et al.* (1984)

Table 7.1 *(Cont)*

Scheme 7.10	
59 (gliocladic acid)	
G. virens	Itoh *et al.* (1980b, 1982)
60 (cadalene hydroxy acid)	
G. virens	Turner and Aldridge (1983)
61 (heptelidic acid)	
G. virens	Itoh *et al.* (1980a,b)
	Stipanovic and Howell (1983)
T. koningii M3947	Endo *et al.* (1985)
T. viride	Itoh *et al.* (1980a,b)
Scheme 7.11	
62 (tricho-acorenol, coccinol)	
T. koningii	Huang *et al.* (1995a)
63 (3,4-dihydroxycarotane)	
G. virens IFO 9166	Watanabe *et al.* (1990)
T. virens ATCC 74180	Lee *et al.* (1995c)
64 (3,4,14-trihydroxycarotane-14-oleate)	
T. virens ATCC 74180	Lee *et al.* (1995c)
Scheme 7.12	
65 (trichodermol)	
T. polysporum CMI 40624	Adams and Hanson (1972)
T. sporulosum CMI 104643	Adams and Hanson (1972)
T. virens	Yamamoto *et al.* (1969)
66 (trichodermin)	
T. polysporum CMI 40624	Adams and Hanson (1972)
T. sporulosum CMI 104643	Adams and Hanson (1972)
T. reesei P-12	
(mutant of *T. r.* QM 9414)	Watts *et al.* (1988)
T. viride	Godtfredsen and Vangedal (1965)
T. virens	Yamamoto *et al.* (1969)
67 (harzianum A)	
T. harzianum ATCC 90237	Corley *et al.* (1994)
68 (mycotoxin T2)	
T. lignorum	Bamburg and Strong (1969)
69 (harziandione)	
T. harzianum IMI 311090	Ghisalberti *et al.* (1992)
Scheme 7.13	
70 (lanosta-3,21-diol)	
T. pseudokoningii	Kamal *et al.* (1971)
G. roseum	Lederer (1969)
71 (ergosterol)	
T. hamatum	Hussain *et al.* (1975)
T. polysporum	Adams and Hanson (1972)
T. pseudokoningii	Kamal *et al.* (1971)
T. sporulosum	Adams and Hanson (1972)
72 (pyrocalciferol)	
T. pseudokoningii	Kamal *et al.* (1971)
73 (helvolic acid)	
Gliocladium sp.	Turner and Aldridge (1983)

Table 7.1 *(Cont)*

74 (ergokonin A)	
T. koningii	Augustiniak *et al.* (1991)
75 (ergokonin B)	
T. koningii	Augustiniak *et al.* (1991)
T. viride IFO 31137	Kumeda *et al.* (1994)
Figure 7.6	
76 (viridin)	
G. fimbriatum CMI 101525	Brian *et al.* (1946)
T. viride	Grove *et al.* (1965, 1966)
G. flavofuscum IMI 100714	Avent *et al.* (1993)
G. virens ACC 213	Moffatt *et al.* (1969)
G. virens GL-21	Lumsden *et al.* (1992b)
T. koningii	Berestestkii *et al.* (1976)
T. viride	Golder and Watson (1980)
77 (2-epiviridin)	
G. flavofuscum IMI 100714	Avent *et al.* (1993)
78 (viridiol)	
G. deliquesc. CMI 101523	Hanson *et al.* (1988)
G. fimbriatum CMI 101525	Hanson *et al.* (1988)
T. viride NRRL 1828	Moffatt *et al.* (1969)
G. virens GL-21	Lumsden *et al.* (1992b)
G. virens ACC 213	Moffatt *et al.* (1969)
79 (virone)	
G. virens	Blight and Grove (1986)
80 (epifridelenol)	
T. pseudokoningii	Kamal *et al.* (1971)
Scheme 7.14	
81 (isonitrinic acid F)	
T. hamatum HLX 1379	Brewer *et al.* (1979, 1982)
	Baldwin *et al.* (1991)
82 (dermadin)	
T. hamatum HLX 1360	Brewer *et al.* (1979, 1982)
T. koningii TK-1	Tamura *et al.* (1975)
T. viride UC 4875	Pyke and Dietz (1966)
	Coats *et al.* (1971)
83 (dermadin methyl ester)	
T. polysporum	Jin and Jin (1989)
84 (epoxy diol)	
T. hamatum HLX 1379	Boyd *et al.* (1991)
85 (spirolactone)	
T. hamatum HLX 1379	Baldwin *et al.* (1985a)
86 (diol isocyanide)	
T. hamatum HLX 1379	Baldwin *et al.* (1985a)
87 (epidiol isocyanide)	
T. hamatum HLX 1379	Boyd *et al.* (1991)
88 (isonitrin A)	
T. hamatum IMI 3208	Baldwin *et al.* (1991)
T. hamatum	Fujiwara *et al.* (1978)
T. harzianum IMI 3198	Baldwin *et al.* (1991)

Table 7.1 *(Cont)*

89	(isonitrin B, deoxytrichoviridin)	
	T. koningii TK-163	Tamura *et al.* (1975)
	Trichod. sp. Leo AK 5139	Ollis *et al.* (1980)
	Trichoderma sp.	Nobuhara *et al.* (1976)
	T. viride IFO 8951	Yamano *et al.* (1970)
90	(hydroxy spirolactone)	
	T. hamatum HLX 1379	Boyd *et al.* (1991)
91	(isonitrin C, trichoviridin)	
	T. hamatum IMRL 3200	Fujiwara *et al.* (1982)
	T. koningii IMRL 3201	Fujiwara *et al.* (1982)
		Baldwin *et al.* (1989)
92	(tetrahydroxy isocyanide)	
	T. hamatum HLX 1379	Boyd *et al.* (1991)
93	(MR304A)	
	T. harzianum	Lee *et al.* (1995a)
94	(isonitrin D)	
	T. harzianum	Fujiwara *et al.* (1982)
95	(homothallin I)	Brasier (1971)
	T. koningii	Pratt *et al.* (1972)
96	(homothallin II)	
	T. harzianum	Faull *et al.* (1994)
	T. koningii	Edenborough and Herbert (1988)
97	(amine from homothallin II)	
	T. koningii	Edenborough and Herbert (1988)
98	(formamide from homothallin II)	
	T. koningii	Edenborough and Herbert (1988)
98a	(N,N-dimethylamine from homothallin II)	
	T. koningii	Mukhopadhyay *et al.* (1996)
99	(3-methoxy-5-hydroxy-5-allyl-cyclopentenone)	
	T. album	Strunz *et al.* (1977)
Scheme 7.15		
100	(gliotoxin)	
	G. fimbriatum	Johnson *et al.* (1943)
	G. virens IMI 101525	Kirby *et al.* (1980, 1988)
	G. virens GL-21	Lumsden *et al.* (1992a)
	T. lignorum	Weindling (1934)
	T. hamatum	Hussain *et al.* (1975)
101	(gliotoxin E)	
	G. virens IMI 101525	Kirby *et al.* (1988)
102	(bisdethiobis(methylthio)gliotoxin)	
	G. virens IMI 101525	Kirby *et al.* (1980, 1988)
	G. virens GL-21	Lumsden *et al.* (1992a)
103	(didehydrogliotoxin)	
	G. virens IMI 101525	Kirby *et al.* (1988)
104	(bisdethiobis(methylthio)didehydrogliotoxin)	
	G. virens IMI 101525	Kirby *et al.* (1988)
105	(bis-N-norgliovictin)	
	G. virens IMI 101525	Kirby *et al.* (1988)
	G. deliquescens	Hanson and O'Leary (1981)

Table 7.1 *(Cont)*

106	(phenol)	
	G. virens IMI 101525	Kirby *et al.* (1988)
107	(cyclo-(glycyl-O-3-methylbut-2-enyl-L-tyrosyl)	
	G. virens IMI 101525	Kirby *et al.* (1988)
	G. deliquescens	Hanson and O'Leary (1981)
108	(3-methylbut-2-enyl ether)	
	G. virens IMI 101525	Kirby *et al.* (1988)
109	(3-hydroxymethylbut-2-enyl ether)	
	G. virens IMI 101525	Kirby *et al.* (1988)
	G. deliquescens	Hanson and O'Leary (1981)
Figure 7.7		
110	(gliovirin)	
	G. virens	Howell and Stipanovic (1983)
111	(cyclo-(L-Pro-L-Leu))	
	T. koningii	Huang *et al.* (1995a)
112	(verticillin A)	
	Gliocladium sp. SCF-1168	Chu *et al.* (1995)
113	(homoverticillin A)	
	Gliocladium sp. SCF-1168	Chu *et al.* (1995)
114	(hydroxyhomoverticillin A)	
	Gliocladium sp. SCF-1168	Chu *et al.* (1995)
115	*G. deliquescens*	Turner and Aldridge (1983)
Figure 7.8		
116	(trichopolyn I)	
	T. polysporum TMI 60146	Fujita *et al.* (1981)
117	(trichopolyn II)	
	T. polysporum TMI 60146	Fujita *et al.* (1981)
Figure 7.9		
118	(3-hydroxy-3,4-dimethylpentanoic acid)	
	G. deliquescens	Hanson and O'Leary (1981)
119	(uracil)	
	T. harzianum	Huang *et al.* (1995a)
120	(melanoxadin)	
	T. harzianum	Lee *et al.* (1995b)
	Trichoderma sp.	Hashimoto *et al.* (1995)
121	(ceramide)	
	T. harzianum	Huang *et al.* (1995a)
122	(valinotricin)	
	T. polysporum	Fujita *et al.* (1984)
123	(melanoxazal)	
	Trichoderma sp. ATF	Takeahashi *et al.* (1996)

secondary metabolite is given in Table 7.1, together with literature references, and Table 7.2 contains those metabolites for which biological activity has been established. The entries in the tables are meant to be illustrative rather than exhaustive. Aspects of the chemistry and biology of *Hypocrea, Trichoderma* and *Gliocladium* species (Taylor, 1986), in which the early work on these species is described, and of the antifungal antibiotics produced by *Trichoderma* species (Ghisalberti and Sivasithamparam, 1991) have been published.

Table 7.2 Biological activities of secondary metabolites solated from *Trichoderma* and *Gliocladium* species

Metabolite	Antibiotic activity	Reference	Other activity
1			Antimutagenic
5	Antiviral, bactericide	Duke (1992)	
	Fungicide	Duke (1992)	
6			Cytotoxic
7			Cardiotonic
13–15	Antifungal	Harris *et al.* (1993)	Squalene synthase inhibitor
18			Phytotoxin
19	Antibiotic	Pettersson (1964, 1965)	
20	Antibiotic	Brian *et al.* (1951)	
21	Antibiotic	Pettersson (1964, 1965)	
22	Antibiotic	Brian *et al.* (1951)	
23	Antibiotic	Brian *et al.* (1951)	
24–26	Antibiotic	Pettersson (1964, 1965)	
28	Bactericide	Duke (1992)	
29	Antiseptic, viridicide	Duke (1992)	Cytotoxic
32	Antibacterial	Adachi *et al.* (1983)	
33			Phosphodiesterase inhibitor
37		Gao *et al.* (1995)	Inhibitor tumor necrosis factor
38	Antibiotic	Nair and Carey (1975)	
40	Antifungal	Dickinson *et al.* (1989)	Plant growth regulator
41	Antifungal	Almassi *et al.* (1991)	
42	Antifungal	Almassi *et al.* (1991)	

Table 7.2 *(Cont)*

Metabolite	Antibiotic activity	Reference	Other activity
43	Antimicrobial	Sawa *et al.* (1994)	
44a			Hypercholesterimic
45	Antifungal	Merlier *et al.* (1984)	Plant growth regulator
	Antimicrobial	Cutler *et al.* (1986)	
46	Antifungal	Claydon *et al.* (1987)	
47–48	Antifungal	Hill *et al.* (1995)	
49–53	Antifungal	Ghisalberti and Rowland (1993)	
51, 53			Plant growth regulator
49, 54			Plant growth regulator
56			Plant growth regulator
59	Antibiotic	Itoh *et al.* (1982)	Antitumor
61	Antibacterial	Itoh *et al.* (1980a,b)	
	Antibiotic	Endo and Karbe (1981), Endo *et al.* (1985)	Antihypercholesteric
63	Antifungal	Watanabe *et al.* (1990)	
64			K channel agonist
65			Mycotoxin
			Antitrichomonal
66	Antifungal	Godtfredsen and	Antitrichomonal
		Vangedal (1965)	Mycotoxin
67	Antifungal	Corley *et al.* (1994)	
68	Antifungal	Bamburg and Strong (1969)	Mycotoxin
73	Antibiotic	Turner and Aldridge (1983)	
74	Antifungal	Augustiniak *et al* (1991)	
75	Antifungal	Augustiniak *et al.* (1991)	

Table 7.2 *(Cont)*

Metabolite	Antibiotic activity	Reference	Other activity
76	Antibiotic	Grove *et al.* (1965)	Inhibitor fungal spore germination
78			Phytotoxic
81–99	Antibiotic	Edenborough and Herbert (1988)	
82	Antibiotic	Brewer *et al.* (1979)	
		Tamura *et al.* (1975)	
		Pyke and Dietz (1966)	
		Coats *et al.* (1971)	
83			Immunosuppressive
93			Inhibits melanin synthesis
95–96		Anderson *et al.* (1978)	Induction of oospores
98a	Antifungal	Mukhopadhyay *et al.* (1996)	
100	Antibiotic	Haraguchi *et al.* (1996), Weindling (1937, 1941)	Acetolactate synthase inhibitor
	Antiviral	Betina (1989)	Immunomodulator
101	Antibiotic	Betina (1989)	
102, 104		Okamoto *et al.* (1986)	PAF inhibitor
103	Antibiotic, antiviral	Betina (1989)	
110	Antibiotic	Howell and Stipanovic (1983)	
112–114			Antitumor activity
116	Antibiotic	Fujita *et al.* (1981)	Immunosuppressive activity
117	Antibiotic	Fujita *et al.* (1981)	
120			Melanin biosynthesis inhibitor
123			Melanin biosynthesis inhibitor
124–125			HIV inhibitor

Figure 7.1 Secondary metabolites not derived from acetate.

7.3.1 Secondary metabolites not derived from acetate

This group of secondary metabolites includes those that arise from carbohydrate metabolism and from the shikimic acid pathway. In fungi, glucose is converted to pyruvate by two major pathways, the Embden-Meyerhof and the pentose phosphate sequences. The only metabolite identified from *Trichoderma* and *Gliocladium* that arises from these sequences is mannitol (**1**) (Figure 7.1), the reduced form of fructose, which has been specifically isolated from *T. hamatum* (Bon.) Bain. (Hussain *et al.,* 1975) and *T. pseudokoningii* Rifai (Kamal *et al.,* 1971). Mannitol is a common metabolite found in fungi, where it is believed to function as a storage molecule. Tartronic acid (**2**), 3-hydroxymalonic acid, found in *T. pseudokoningii* (Kamal *et al.,* 1971) may originate from hydrolysis and oxidation of 2-phosphoglycerate, an intermediate in the Embden-Meyerhof sequence.

The main role of the shikimic acid pathway is that of producing the amino acids phenylalanine, tyrosine and tryptophan. It has its origin in the condensation of erythrose-4-phosphate from the pentose phosphate pathway and phosphoenolpyruvate from the Embden-Meyerhof pathway. The central intermediate is the cyclic polyol shikimic acid which is the precursor of carbomonocyclic aromatic compounds. This pathway also does not appear to lead to significant accumulation of secondary metabolites in the two genera under consideration. The simple compounds methyl benzoate (**3**), *p*-hydroxybenzyl alcohol (**4**) from *T. hamatum* (Huang *et al.,* 1995a) and ferulic acid (**5**) from *G. virens* (Dickinson *et al.,* 1995) probably arise from shikimic acid. 2,5-Dimethoxybenzoquinone (**6**), isolated from *T. pseudokoningii* (Kamal *et al.,* 1971) is possibly derived from shikimic acid. Dihydrocoenzyme Q10 (**7**) and small amounts of coenzyme Q10 (**8**) have been isolated from *G. roseum* Bain. (Bentley and Lavate, 1965). These compounds arise by contributions from two biosynthetic pathways: the quinone portion is derived from shikimic acid (Bentley and Lavate, 1965) and the terpenoid side chain from mevalonic acid.

7.3.2 Secondary metabolites derived from the tricarboxylic acid cycle (TCA)

The TCA cycle completes the oxidation of glucose to carbon dioxide in the cell and provides intermediates for biosynthesis of amino acids and of some secondary

Figure 7.2 Secondary metabolites derived from TCA cycle.

metabolites. As mentioned previously, some TCA intermediates, e.g. citric acid in *Aspergillus,* may accumulate in non-physiological quantities under certain conditions.

Succinic acid **(9)** (Figure 7.2), an intermediate from the TCA cycle, has been isolated from *T. pseudokoningii* together with itaconic acid **(10)** (Kamal *et al.,* 1971). This second compound has been shown to arise from decarboxylation of *cis*-aconitic acid, another intermediate in the TCA cycle. The imide pencolide **(11)**, reported to be produced by a species claimed to be *T. album* (Ren, 1977), could be generated from the condensation of citraconic acid (*Z*-2-methyl-2-butene dioic acid) and the amino acid threonine.

A number of fungal metabolites appear, formally at least, to arise from condensation of an acetate-derived chain with TCA cycle intermediates. Carolic acid **(12)** has been isolated from a *Trichoderma* sp. and it has been shown to exist as a mixture of *E* and *Z*-isomers in solution (Turner and Aldridge, 1983).

More obvious is the biosynthetic derivation of the viridiofungins A, B and C **(13–15)** isolated from a strain of *T. viride* Pers.: Fr. (Harris *et al.,* 1993). These compounds appear to arise from precursors generated in three different pathways. The citric acid moiety, which is clearly visible, has undergone condensation with a C16 fatty acid CoA unit and amide formation with the three aromatic amino acids phenylalanine, tyrosine and tryptophan. The viridiofungins represent the first examples of this type of compound to be isolated from either *Trichoderma* or *Gliocladium,* although they are also produced by some *Aspergillus* and *Penicillium* species.

7.3.3 Fatty acids

Normal fatty acids are derived from the linear condensation of acetyl CoA (starter unit) and malonyl CoA (extending unit; normally delivered to an acyl carrier

Acetyl CoA
Malonyl ACP
Acetoacetyl ACP
(malonyl CoA)$_{n+1}$
n = 5 PALMITIC ACID
n = 6 STEARIC
n = 7, m = 1 OLEIC ACID
n = 4, m = 2 LINOLEIC ACID
n = 1, m = 3 LINOLENIC ACID

Scheme 7.1 Biosynthesis of fatty acids.

protein, ACP). After each addition step, a sequence of reactions results in the removal of the ketone functionality and mainly saturated fatty acids are produced (Scheme 7.1). Unsaturation, leading to oleic, linoleic and linolenic acids with *Z*-configuration of the double bonds, occurs after formation of the chain. The most abundant fatty acids in fungi are palmitic (C16), stearic (C18), oleic (C18; ?1), and linoleic acid (C18; ?2), but small amounts of other common saturated and unsaturated straight-chain acids (C14–C24) are also produced. Most commonly, these compounds can be obtained by extraction of the mycelium and are essentially not restricted to any particular species. Their classification as secondary metabolites is arguable. Palmitic acid has been isolated from *T. hamatum* (Hussain *et al.,* 1975) and *T. koningii* Oudem (Benoni *et al.,* 1990). A mixture of fatty acids containing mostly palmitic, stearic, oleic, linoleic acids and a C24 fatty acid was isolated from a strain of *T. harzianum* (Ghisalberti *et al.,* 1990). The triply unsaturated fatty acid ester, methyl-2,4,6-octatriene carboxylate **(16)** (Figure 7.3), from *T. pseudokoningii* (Kamal *et al.,* 1971) is a more likely secondary metabolite. Also isolated from this strain was the triacetylene trichodermene A **(17)** which, if the structure assigned is correct, represents the first branched-chain polyacetylene from a natural source. Polyacetylenes normally arise by sequential desaturation of alkenes.

$H_3C\text{-}(CH{=}CH)_3\text{-}CO_2CH_3$

16

17

Figure 7.3 Secondary metabolites derived from fatty acids.

7.3.4 Polyketides

Polyketides constitute the largest class of fungal secondary metabolites. The large array of structurally diverse polyketides produced by microorganisms all originate from repetitive connections of short chain fatty acids, e.g. acetate or propionate, by

Scheme 7.2 Biosynthesis of polyketides.

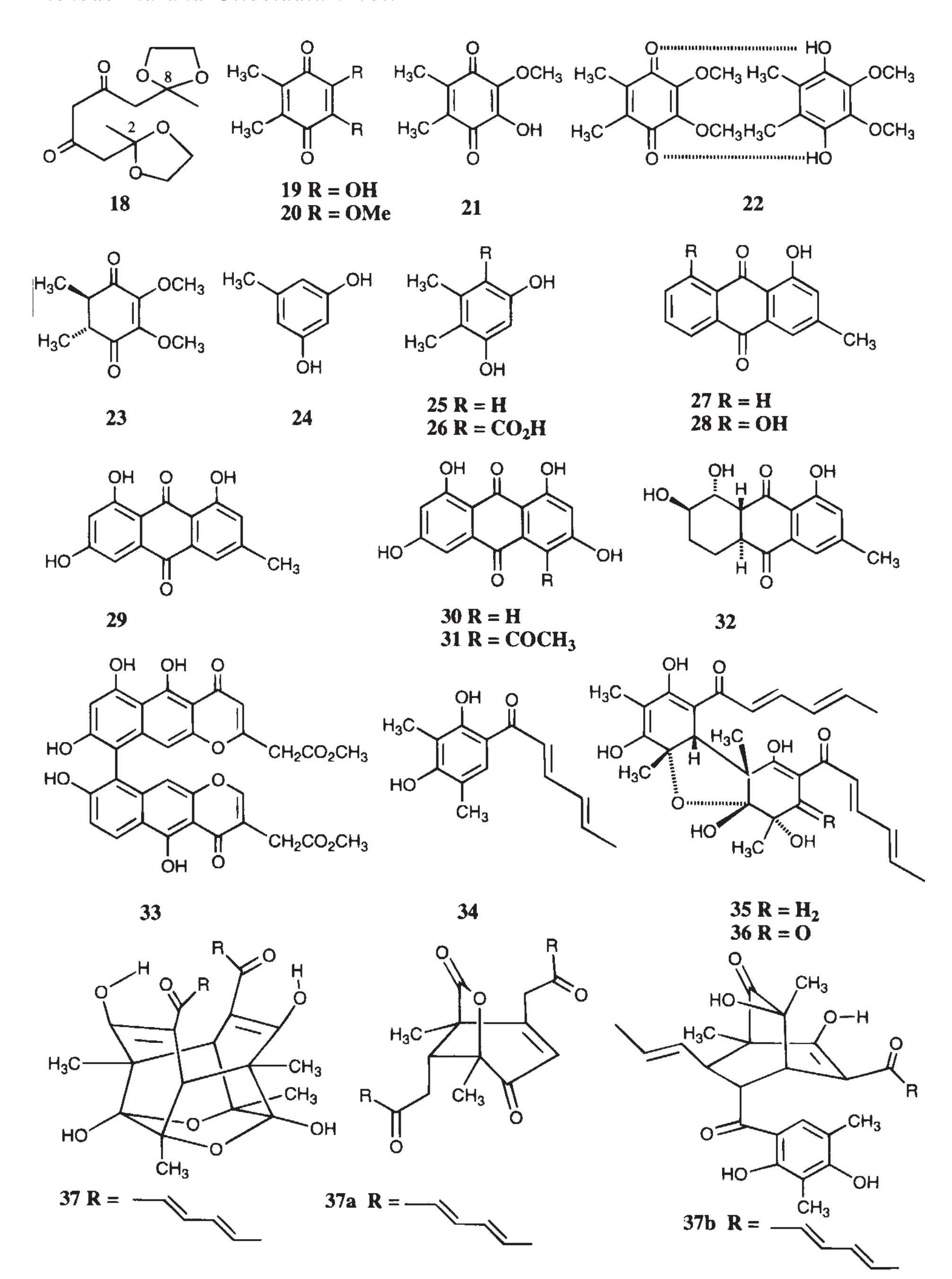


Figure 7.4 Polyketide-derived secondary metabolites.

pathways very similar to those of fatty acid biosynthesis (Scheme 7.2). However, the polyketide synthases (PKS) can by-pass the reductive steps of the fatty acid synthase and the oxidation level of the growing polyketide chain is adjusted after each condensation step to generate a carbon skeleton containing keto, hydroxy,

olefinic or fully reduced carbons. The intermediate produced remains bound to the synthase complex until chain extension is complete. After the product is released from the protein, separate enzymes may operate on the product to further transform it, by cyclisation, oxidation and alkylation, to produce the final metabolite. In the simplest case, the distal ketone group of the first formed keto ester, the acetoacetyl S-complex, is not reduced before addition of the next malonyl unit. The overall result is that a polyketone ester is produced (Scheme 7.2) which can cyclise in a number of ways, two of which are shown for the folded arrangements **A1** and **A2** of a tetraketide. In general, tetraketides and higher ketides cyclise to produce an aromatic system which can then undergo a number of modifications.

The simplest polyketide metabolite reported for these species appears to be the 2,8-bis-ethylene ketal of 2,4,6,8-nonanetetrone **(18)** (Figure 7.4), isolated from G. *zaleskii* (Nadkernichnaya *et al.*, 1986). The presence of the ketal functions is unusual and it is more likely that the corresponding polyketide, 2,4,6,8-nonanetetraone, is the true natural product. This could arise from decarboxylation of the pentaketide containing a terminal carboxylic acid.

A number of polyketide-derived metabolites have been isolated from *Trichoderma* and *Gliocladium* species (Figure 7.4). The benzoquinones **(19, 20, 21, 23)** and the quinhydrone **(22)** were first obtained from G. *roseum* (Brian *et al.,* 1951; Pettersson, 1964, 1965). It is likely that the quinones are artefacts and arise from the corresponding hydroquinones by air oxidation during isolation. Although considerable work has been carried out to elucidate the exact sequence of biosynthetic steps involved in the formation of these compounds, this point has not yet been fully resolved (Turner, 1971; Turner and Aldridge, 1983). A possible sequence, which also considers the involvement of the simple aromatic metabolites orcinol **(24)**, 3,5-dihydroxy-1,2-dimethylbenzene **(25)** and 2,3-dimethyl-4,6-dimethoxybenzoic acid **(26)** is presented in Scheme 7.3 (SAM is the acronym for S-adenosyl methionine).

The anthraquinone pigments **27–29** (Figure 7.4) are normal products that are formed from octaketides and their biosynthesis is unexceptional (prefolding arrangement **B1,** Scheme 7.2). A strain of *T. polysporum* when challenged with *Fomes annosus* (= *Heterobasidion annosum* (Fr.: Fr.) Bref.) produced the pigments **27–29** which had antagonistic effect to *F. annosus* (Donnelly and Sheridan, 1986). Interestingly, a conidiating mutant of *T. viride,* obtained after exposure to UV irradiation, produced the anthraquinones (**30** and **31**) instead of the usual ones **(27–29)**. In these fungi, conidiation is inducible by light and the accumulation of pigments in the culture medium and mycelium begins after the onset of conidiation (Betina, 1995). Pigment formation in culture grown in the dark was low. The significance of this is that, although both sets of anthraquinones are derived from octaketides, each reflects different folding patterns of the precursor (**B1** and **B2,** Scheme 7.2). Presumably in the mutants, one or more of the enzymes required for production of **27–29** was rendered inoperative, thus allowing an alternative pathway to be expressed. In the original organism, this latter pathway was not activated to a significant extent. Of some biosynthetic interest is the modified quinone **(32)**, the first example of its type, which is produced in small quantities by a strain of *Trichoderma*. Greater amounts of this metabolite are produced when the *Trichoderma* strain is challenged by *Fusarium* spp. grown in combined culture (Adachi *et al.,* 1983).

The dimeric xanthone **(33)** from a *Trichoderma* sp. is interesting since the monomeric unit arises from yet another folding pattern available to the octaketide previously implicated in the formation of the anthraquinones (**B3,** Scheme 7.2).

Scheme 7.3 Hypothetical biosynthetic correlations between aromatic compounds and quinones (SAM = S-adenosylmethionine).

Various modifications can occur in the process of the polyketide assembly. One of these is that reactions normally associated with fatty acid biosynthesis can occur. For example, one or more oxygens of the polyketide units can be eliminated after reduction to an alcohol with resultant introduction of double bonds. This appears to be the case for the compound **(34)**, produced by a strain of *T. longibrachiatum* (Andrade *et al.,* 1992), which can be regarded as an acylphloroglucinol of hexaketide origin and which has cyclised according to the folding arrangement **A2** (Scheme 7.2). The dimerization of this compound to compounds **35–37** (Andrade *et al.,* 1996) is not unexpected and is well recognized for anthraquinone metabolites from *Penicillium* species.

7.3.5 Oxygen heterocyclic compounds

This class is represented by a small group of compounds, the biosyntheses of which have been studied in some detail. Nectriapyrone **(38)** and vermopyrone **(39)** (Scheme

Scheme 7.4a Biosynthesis of nectriapyrone **(38)** and vermopyrone **(39)**.

Scheme 7.4b Biosynthesis of harzianopyridone **(40)** (SAM = S-adenosylmethionine).

7.4a) have been isolated from *G. vermoesenii* (Biourge) Thom (= *Penicillium vermoesenii* Biourge) (Avent *et al.,* 1992a). Whereas vermopyrone was a new compound, nectriapyrone had previously been isolated from *Gyrostroma missouriensis* Naumov. Originally, it had been suggested that nectriapyrone was a terpene arising via the isoprenoid pathway from mevalonic acid. Biosynthetic studies with acetate, singly-and doubly-labelled with ^{13}C, and with methionine (Avent *et al.,* 1992b) clearly established that, in fact, it was a tetraketide that had undergone methylation with S-adenosyl methionine as shown in Scheme 7.4a. The labelling pattern in vermopyrone showed it to be a catabolic product of nectriapyrone. Similar labelling studies on harzianopyridone **(40)** from *T. harzianum* (Dickinson *et al.,* 1989) indicated that it also was derived from a tetraketide with the possible involvement of aspartic acid (Scheme 7.4b). It is interesting to note that **40,** initially obtained in racemic form, has also been isolated from a different strain in the laevorotatory form (Cutler and Jacyno, 1991), although the optical purity of the latter sample has not been determined. Racemic **40** has significant antifungal activity, whereas (–)-**40** has only weak antifungal and antibacterial activity. On the other hand, the latter exhibits plant growth regulating

properties. These observations suggest that the two enantiomers may possess different activities.

The rather unusual butenolide harzianolide **(41)** (Scheme 7.5) has been isolated from strains of *T. harzianum*. The first report of its occurrence (Almassi *et al.*, 1991) was ignored by two other groups, each of which claimed it as a new compound (Claydon *et al.*, 1991; Ordentlich *et al.*, 1992). From the biosynthetic view, the interesting features of harzianolide are that although it has the appearance of a polyketide-derived metabolite, it possesses an odd number of carbons and both

Scheme 7.5 Hypothetical biosynthetic schemes for harzianolide **(41)** and harzianic acid **(43)**.

chains have a terminal methyl group. Moreover, the butenolide ring is formed from two contiguous branches along the chain which are unlikely to originate from S-adenosyl methionine since methylation rarely occurs on adjacent carbon atoms. The biosynthesis of **41** was investigated using [1-^{13}C]-, [2-^{13}C]- and [1,2-^{13}C2]acetate and the results showed that it was derived from seven acetate units with loss of one carbon. A rationale for the labelling pattern observed is shown in Scheme 7.5 (Avent *et al.,* 1992c) and relies on a precedent encountered in the biosynthesis of aspyrone, a metabolite of *Aspergillus melleus* Yukawa (Staunton and Sutkowski, 1991). In the original work on harzianolide, the related compound **42** was also isolated (Almassi *et al.,* 1991), but this may simply be an artifact of the isolation procedure.

Other metabolites that are probably of polyketide origin have been isolated from strains of *T. harzianum*. The tetramic acid derivative harzianic acid **(43)** (Scheme 7.5) was isolated from a water sample from the Hiroshima prefecture in Japan (Sawa *et al.,* 1994). Although no studies of its biosynthesis have been carried out, it appears that the compound arises from a pentaketide that has undergone condensation with an amino acid.

Trichoharzin **(44)** (Scheme 7.6) has been isolated from a culture of a strain of *T. harzianum* found on the fresh marine sponge *Micale cecilia* (Kobayashi *et al.,* 1993). Cultivation of the strain in either fresh water medium or salty water medium gave significantly different profiles of metabolites. Extraction of the salty water medium

Scheme 7.6 Hypothetical biosynthetic scheme for trichoharzin **(44)**.

yielded **44**. This compound is probably an octaketide that has undergone a putative Diels-Alder reaction (Scheme 7.6) similar to that presumed for the biosynthesis of lovastatin, a metabolite from *Aspergillus terreus* Thom (Witter and Vederas, 1996) that shares some similarity with **44**. The claim that strains of *T. longibrachiatum* and *T. pseudokoningii* produce compactin **(44a)**, more commonly obtained from *Penicillium citrinum* Thom and *P. brevicompactum* Dierckx, is of some significance given the ability of this and related compounds to lower LDL cholesterol in patients with hypercholesterolemia (Endo and Hasumi, 1993).

7.3.6 Pyrones

One of the first volatile antifungal compounds isolated from *Trichoderma* species was 6-pentyl-*a*-pyrone (6PP; **45**) (Figure 7.5). This compound is a non-toxic flavouring agent that was synthesised for industrial purposes before its discovery as a natural product. The first identification of this compound in *T. viride* was made by Collins and Halim (1972). Subsequently, it has been isolated from several *Trichoderma* species and strains (Table 7.1) (Dickinson, 1993). 6PP is in fact the metabolite responsible for the "coconut aroma" associated with strains of *Trichoderma* (Bisby, 1939) and in particular those that showed appreciable inhibition in the growth of *Rhizoctonia solani* Kühn (Dennis and Webster, 1971). The biosynthesis of 6PP, and indeed of all compounds in this group, is a matter for conjecture. An attempt has been made to determine its origin from linoleic acid but the results were inconclusive since the radioactivity incorporated from linoleic acid into 6PP was not shown to be derived directly from linoleic acid (Serrano-Carreon

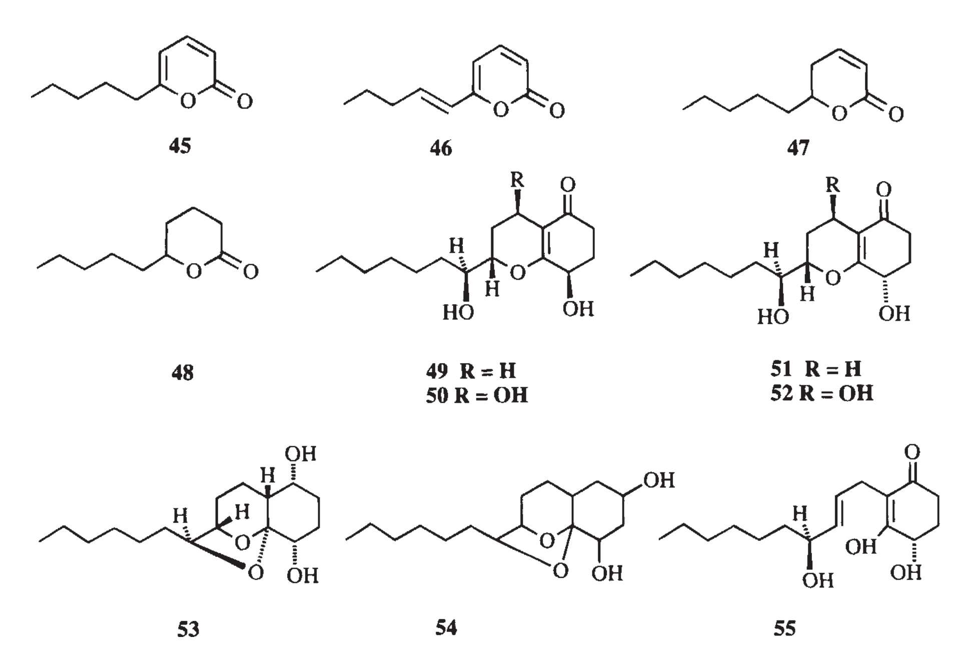


Figure 7.5 Pyrone secondary metabolites.

Scheme 7.7 Hypothetical sequences for the biosynthesis of pyrones.

et al., 1993). Whilst this route remains a possibility, we prefer to consider these compounds to be derived from a polyketide pathway as indicated in Scheme 7.7.

The related dehydroderivative **(46)** also has the characteristic coconut odour and was isolated during a search for the volatile metabolite of *T. viride* and *T. koningii* responsible for inducing oospore formation in cultures of the A_2 isolates of *Phytophthora cinnamomi* Rands (Moss *et al.,* 1975). More recently, a patent describing the use of *Trichoderma* as a microbiological control agent refers to massoilactone **(47)** and *d*-decanolactone **(48)** as metabolites of *Trichoderma* species (Hill *et al.,* 1995). These compounds have also been isolated from *T. viride* (Nago *et al.,* 1993). Compounds **45–48** may be considered as pentaketides whose derivation can be rationalised in a number of ways, some of which are as shown in Scheme 7.7. There is some evidence that the deceno-*d*-lactone **(48)** can be transformed

into **45** and **47** (Nago *et al.,* 1993). These simple pyrones are matched by a group of octaketides **49–55** (Figure 7.5) that have been isolated from strains of *T. koningii* and *T. harzianum* (Table 7.1). Two possible modes of derivation are illustrated in Scheme 7.7. The relative stereochemistry of **49–54** has been assigned on the basis of spectroscopic studies. Recent attempts at determining the absolute stereochemistry have led to contradictory results that particularly concern the relative and absolute stereochemistry of **53** (Mori and Abe, 1995; Xu and Zhu, 1995). The evidence for the structure of **54** is not strong and no indication of stereochemistry has been given (Parker *et al.,* 1995a). Interestingly, a suite of compounds from which a representative **(55)** was purified may reflect a type of precursor in the biosynthetic pathway that leads to **49–54** (Ghisalberti and Rowland, 1993).

7.3.7 Terpenoids

Terpenes are the most abundant and most widely distributed secondary metabolites in nature. They occur in a remarkable variety of structural types and best illustrate the multiplicity of processes available in secondary metabolic pathways. The generally accepted view of their biosynthesis is that they all arise from mevalonic acid via the intermediates isopentenyl and dimethylallyl diphosphate which combine to form geranyl diphosphate (a monoterpene) (Scheme 7.8). Sequential addition of further

Scheme 7.8 Biosynthesis of acyclic terpenes.

isopentenyl diphosphate units generates farnesyl (sesquiterpene), geranylgeranyl diphosphate (diterpene) and sesterpene diphosphate (C25). Also possible is the head-to-head combination of farnesyl diphosphate to give squalene (C30; triterpene) and the C40 intermediate (tetraterpene). Each of these intermediates can then undergo a myriad of cyclization events.

As yet, no individual monoterpene has been isolated from either of the two genera, although the formation of volatile terpenes, lactones and alcohols from *Trichoderma* species has been indicated (Zeppa *et al.,* 1990). In general, monoterpenes are relatively volatile and may easily escape detection unless special precautions are taken. On the other hand, the frequent detection of 6PP, also a volatile compound, would suggest that, if monoterpenes are produced, they are produced in small amounts.

Sesquiterpenes

The biosynthesis of sesquiterpenes is controlled by sesquiterpene synthases and, together, these enzymes are responsible for the formation of more than 200 distinct carbon skeletons. Each synthase can convert the universal precursor, farnesyl diphosphate, to a distinct sesquiterpene via the common mechanism of ionization of the allylic diphosphate ester followed by a precise sequence of intramolecular electrophilic addition reactions (Cane and Xue, 1996). One of the functions of a synthase is that of imposing a particular folding conformation on the acyclic precursor farnesyl diphosphate or its equivalent nerolidyl diphosphate. The number of sesquiterpenes with different skeletons isolated from *Trichoderma* and *Gliocladium* species, to some extent, illustrates this variability.

The biosynthetically simplest examples are the cyclonerodiols **(56–58)** (Scheme 7.9) from *T. koningii* and *T. harzianum.* Their formation can be rationalised as arising from nerolidyl diphosphate as shown in Scheme 7.9. Evidence for this pathway has been obtained with cell-free extracts from *G. fujikuroi* and a similar sequence of events in *Trichoderma* can be assumed. Interestingly, there is some evidence that **56** is not metabolically stable since it can be detected in the culture broth after 8 days but not after 14 days (Cutler *et al.,* 1991a). However, in *T. harzianum* it can still be isolated from a 3-month-old culture (Ghisalberti and Rowland, 1993).

Scheme 7.9 Biosynthesis of cyclonerodiols.

This difference may very well reflect the differences in activity in the two strains of an oxidase which converts **56** into what would be an unstable epoxide.

Of some interest are the monocyclic and bicyclic compounds **59–62**. The compounds **59–61** can be envisaged to be derived from the sequence shown in Scheme 7.10. It should be mentioned that the evidence for structure **60** is lacking (Turner and Aldridge, 1983), but it appears to be an acceptable structure insofar as it is predicted from a consideration of the likely biosynthetic pathway (Scheme 7.10).

Tricho-acorenol **(62)**, isolated from a *T. koningii,* has been claimed to be a new compound. It is in fact identical to coccinol previously isolated from *Fusidium coccineum* Fuckel in 1968 (Visconti Di Modrone, 1968). Its biosynthesis in this organism has been studied (Godtfredsen, 1978) and the more pertinent details are presented in Scheme 7.11.

The two daucane sesquiterpenes **(63, 64)**, isolated from *G. virens* and *T. viride,* belong to a relatively rare group of compounds which, to a large extent, are characteristics of the Umbelliferae family of plants (Ghisalberti, 1994). The biosynthesis of **63** has been investigated and appears to conform to that assumed to operate in plants (Scheme 7.11).

The group of compounds **65–68,** isolated from several *Trichoderma* species, belongs to the trichothecenes class of sesquiterpenes. This class has attracted much attention because of the likely involvement of its members in mycotoxicoses of farm animals (Grove, 1988). These compounds illustrate yet another way in which farnesyl diphosphate can cyclise (Scheme 7.12). While mostly produced by *Fusarium* species, they have also been isolated from the unrelated species of *Dendrostilbella, Myrothecium, Stachybotrys* and *Trichothecium* (Grove, 1988).

Scheme 7.10 Biosynthesis of cadinane sesquiterpenes.

Nerolidyl diphosphate

62

63 R = H
64 R = OLEOYL

Scheme 7.11 Biosynthesis of acorenol **(62)** and the daucanes **(63, 64)**.

It is important to realise that consideration of the biosynthesis of these compounds can very often be useful in predicting the structure of new compounds belonging to a particular class. Since, as shown in the various schemes, a pathway involves many steps, there is the possibility of premature deprotonation of the various cationic intermediates occurring in competition with the normal pathway. For example, simple deprotonation of the first formed cyclic intermediate in the production of **60** (Scheme 7.10) would produce a macrocyclic triene and/or oxygenated derivatives from it.

Diterpenes

The only diterpene isolated so far from the two species is harziandione **(69)** from *T. harzianum* (Scheme 7.12). Harziandione was isolated from the strain that also produces the antifungal compounds **49–53** (Figure 7.5) but does not seem to have any significant antifungal activity. The complexity of this compound, which contains a four-, five-, six- and seven-membered ring, suggests an elaborate biosynthetic pathway. On this basis, it seems likely that, in *T. harzianum* at least, the probability of finding other diterpene metabolites is high.

Recently, a metabolite with fungal growth inhibitory activity was isolated from a strain of *T. viride* isolated from a soil sample in Sardinia. The structure assigned

Nerolidyl diphosphate

65 R = H
66 R = Ac

67

68

69

Scheme 7.12 Steps in the biosynthesis of trichothecenes.

from detailed spectroscopic studies was that corresponding to an isomer of harziandione **(59)** (Mannina *et al.,* 1997). We have compared the proton and ^{13}C-NMR spectral data and have concluded that it is in fact identical to harziandione.

Triterpenes and sterols

A number of triterpene-derived compounds have been found in the two genera. The first formed tetracyclic triterpene on the pathway from squalene to the sterols is lanosterol in fungi and animals (Scheme 7.13) and cycloartenol in algae and plants. The conversion of lanosterol to the sterols includes losses of the C-4 and C-14 methyls, the order in which these transformations occur being dependent on the organism and the prevailing environmental conditions (Turner and Aldridge, 1983). Lanostadiol **(70),** ergosterol **(71)** and pyrocalciferol **(72)** (Scheme 7.13) have been

Scheme 7.13 Biosynthesis of sterols.

isolated from *T. pseudokoningii* (Kamal *et al.,* 1971). Ergosterol is the most commonly occurring fungal sterol. Helvolic acid **(73)** which has been identified in a *Gliocladium* species (Turner and Aldridge, 1983) represents an alternative route of cyclisation to that which leads to lanosterol. The highly oxygenated ergosterol derivatives, ergokonin A and B **(74, 75)**, are produced by a *T. koningii* isolate. Ergokonin A is characterised by being esterified at the 3-hydroxyl with an unusual 3-hydroxy-leucine-3-O-sulphate moiety (Augustiniak *et al.,* 1991).

The viridin family of steroidal antibiotics (Hanson, 1995), a group of furanosteroids, were first discovered in *Gliocladium* species with the isolation of viridin **(76)** from *G. virens* (Brian and McGowan, 1945; Brian *et al.,* 1946) (Figure 7.6). The epimer **(77),** which is readily formed under basic conditions, is sometimes isolated along with viridin from *G. flavofuscum* Miller *et al*. (Avent *et al.,* 1993) but is probably an artifact of the isolation procedure. Viridiol **(78)** has been obtained from G. *fimbriatum* (Moffatt *et al.,* 1969), *G. virens* and *G. deliquescens* Sopp. (= *G. viride* Matr.) (Howell and Stipanovic, 1984). Interestingly, virone **(79)** was obtained as a minor metabolite from a culture of a strain of *G. virens* that had been grown at 32°C (Blight and Grove, 1986). Its structure suggests that it may be an intermediate in the biosynthesis of the viridins which has been the subject of considerable study

Figure 7.6 Viridins and triterpene **(80)**.

(Hanson, 1995). The labelling pattern of viridin biosynthesised from [2-^{14}C]-mevalonic acid was consistent with a triterpene/sterol rather than a diterpene origin. This was supported by the incorporation of lanosterol into viridin, and other biosynthetic studies indicated the intermediacy of a lanostane rather than a cycloartenol precursor, which would have been characteristic of plant biosynthesis. Moreover, the isolation of the simple hydroxy acid **(118)** (see Figure 7.9), which represents the side chain of a steroid precursor, from *G. deliquescens* (Hanson and O'Leary, 1981) suggests the intervention of an ergostane rather than a cholestane side chain. This strongly suggests that the cleavage of the side chain follows a sequence characteristic of mammalian systems rather than the stepwise degradation observed for bacterial systems. Compounds of the viridin class have also been isolated from *Myrothecium roridum* Tode ex Fries, *Nodulisporum hinnuleum* G.Smith, *Penicillium funiculosum* Thom, and *P. wortmanni* Kloeker (Hanson, 1995).

The pentacyclic triterpene epifriedelenol **(80)** has been isolated from *T. pseudokoningii* (Kamal *et al.,* 1971) and its presence indicates that biosynthetic pathways more commonly associated with higher plants are also available to *Trichoderma*.

7.3.8 Metabolites derived from amino acids

Isocyano derivatives

The isocyano functional group, with the unique characteristic of a carbon bonded only to a single atom together with the foul odour of its volatile derivatives, has attracted the interest of both theoretical and synthetic chemists. The first naturally occurring isocyano metabolite, xanthocillin, was reported from *Penicillium notatum* in 1956 (Scheuer, 1992). The second microbial metabolite containing such a function, dermadin **(82),** was isolated 10 years later from *Trichoderma* species, although in the meantime some isocyanoterpenoids were isolated, mostly from marine sponges (Edenborough and Herbert, 1988; Scheuer, 1992). The origin of the

L-Tyrosine

81

82 R = H
83 R = CH_3

84

85

86
87 (isomer)

88

89

90

91

92

93

94

95

96 R = NC
97 R = NH_2
98 R = NHCHO
98aR = $N(CH3)_2$

99

Scheme 7.14 Biosynthesis of isocyano metabolites.

Trichoderma isocyano metabolites **(81–99)** (Scheme 7.14) is difficult to ascertain from their structures, but Baldwin *et al.* (1985b) have been able to show that they arise from the amino acid tyrosine. Briefly, the results showed that the side chain of tyrosine is incorporated intact by a cyclization process involving the carboxylic acid group and an aromatic carbon (Scheme 7.14; **A** or **B**), originally *meta-* to the phenolic carbon. All the carbon atoms of tyrosine are retained with the exception

of the carbon adjacent to that bearing the hydroxyl group. The results of these studies together with some conjectural biosynthetic relationships are presented in Scheme 7.14.

The structural studies on this group of metabolites have been fraught with difficulty, largely because of their instability. As a consequence, there is a great deal of confusion in the literature with structures being misassigned, a problem that has been corrected by meticulous synthetic studies (see, for example, Baldwin *et al.,* 1991). With the benefit of hindsight, we have tried to include these corrections in this compilation.

Dermadin **(82)** was first isolated from *T. viride* (Pyke and Dietz, 1966) and its antibiotic activity was the subject of a patent in 1971 (Coats *et al.,* 1971). The same compound was also isolated from *T. koningii* together with trichoviridin **(89)**. Their structures were established from chemical and X-ray crystallographic analyses (Brewer *et al.,* 1979; Ollis *et al.,* 1980). A host of related compounds have subsequently been isolated from strains of *Trichoderma* species during work aimed at identifying the antibiotic activity of these species. Other examples have been identified from studies involving the volatile metabolites of *T. koningii* which induce production of oospores in *Phytophthora cinnamomi* of the A_2 mating type. Homothallin I **(95)** and II **(96)** and the corresponding amine and N-formyl derivative **(97, 98)** have been identified from the culture broth (see Edenborough and Herbert, 1988). Recently, the corresponding N,N-dimethyl analogue **(98a)** has been isolated from *T. koningii* (Mukhopadhyay *et al.,* 1996) after treatment of an unstable precursor, probably the diketone, with dimethylamine. There is a possibility that isonitrin D, assigned structure **94,** is in fact homothallin II (Edenborough and Herbert, 1988). As mentioned previously, earlier attempts to identify metabolites with similar activity from *T. viride* had led to the isolation of the pyrone **(46)** which, however, could not be shown to be the active agent (Moss *et al.,* 1975). The diene-isocyanide **(81)** inhibits cellulose digestion by *Bacterioides succinogenes,* an important component of rumen flora (Liss *et al.,* 1985). A method for the stabilization of these metabolites by formation of a rhodium complex has been developed and these complexes have facilitated the analysis and the purification of these metabolites (Boyd *et al.,* 1991; Hanson *et al.,* 1985).

T. harzianum strains generally do not produce isocyanides, but one subset of this species does have morphological and physical characteristics similar to *T. viride* (Fujiwara *et al.,* 1982; Okuda *et al.,* 1982). Faull *et al.* (1994) have found that a strain of *T. harzianum,* which normally produced 6PP **(45)** when grown in culture, on exposure to UV light generated a methionine-requiring mutant which became a homothallin II producer. This result has been interpreted to mean that there may be a number of "silent" biosynthetic pathways in *Trichoderma* that could be activated by simple mutation.

Diketopiperazines

This group contains compounds derived from cyclic dipeptides that arise by condensation of two *a*-amino acids. Gliotoxin **(100)**, originally isolated from cultures of *G. fimbriatum,* was the first member of what has now become a large group of epidithiodiketopiperazine derivatives known to be produced by fungi. Gliotoxin is generated biosynthetically from L-phenylalanine and L-serine via the cyclic dipeptide **(A)** (Scheme 7.15). Introduction of sulfur from sulfate leads to gliotoxin, probably

Scheme 7.15 Biosynthesis of diketopiperazines.

by means of the arene oxide **(B)**. Interestingly, the introduction of sulfur occurs with formation of the *R,R*-diastereoisomer in *G. virens* and with the formation of the *S,S*-diasteroisomer in *Hyalodendron* spp. (Pita Boente *et al.,* 1991). The conversion of gliotoxin to **102** occurs irreversibly (Kirby *et al.,* 1980). The epitrisulfide **(101)** also occurs in *G. virens* and is commonly found in, among others, *Penicillium terlikowski* Zaleski. Surprisingly, the metabolites **106–109** contain a *para*-hydroxyl group on the aromatic ring and would, at first sight, appear to be derived from tyrosine. However the rationale provided, without any supporting evidence, is that these compounds arise from the cyclization of an arene oxide isomeric to **B** (Hanson and O'Leary, 1981).

A strain of *G. virens* produces the antibiotic gliovirin **(110)** which, although structurally complex, is essentially a diketopiperazine arising from two units of phenylalanine (Figure 7.7, bolded bonds) (Stipanovic and Howell, 1982; Stipanovic *et al.,* 1994). The simpler diketopiperazine **(111)** from *T. koningii* (Huang *et al.,* 1995a) involves amide formation between proline and leucine. A group of metabolites from

112 R = CH_3

113 R = CH_2CH_3

114 R = $CH(OH)CH_3$

Figure 7.7 Other metabolites derived from amino acids.

Gliocladium species **(112–114)** are essentially dimers involving the tryptophan analogue of gliotoxin but, instead of serine, the second amino acid is alanine, α-aminobutyric acid or threonine. These compounds have attracted interest because of their antitumour activity which appears to be operating through the inhibition of *c-fos* proto-oncogene (Chu *et al.,* 1995). They are related to a number of metabolites isolated from *Chaetomium, Verticillium* and *Acrostalagmus* species (Turner and Aldridge, 1983). The quinone **(115)** is probably derived from tryptophan and is related to a group of quinones, the asterriquinones, more commonly produced by *Aspergillus terreus* (Turner and Aldridge, 1983). The contribution of the mevalonic acid pathway can be seen in the two 1,1-dimethylprop-2-enyl substituents on C2 of the indole moiety.

7.3.9 Polypeptides

The **peptaibols** are a family of **pept**ides which contain a high proportion of the unusual amino acid *a,a*-dimethylisobutyric acid **(Aib)**, a C-terminal amino alcoh**ol** and an acylated N-terminal group (Table 7.3). The first compound of this class was isolated from *T. viride* and named alamethicin (Brewer *et al.,* 1987; Meyer and Reusser, 1967). Although obtained as a crystalline material, it was established that alamethicin was a mixture of at least 12 compounds each containing 20 amino acid residues. There are two major forms of alamethicin which differ only in the nature of

Table 7.3 Classes of peptaibols from *Trichoderma* and *Gliocladium* species

	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Alamethicins	Ac-Aib-Pro-Aib-Ala-Aib-**Ala**-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-**Glu**-Gln-Pheol
Suzukacillins	Ac-Aib-Ala-Aib-Ala-Aib-**Ala**-Gln-Aib-**Aib**-Aib-Gly-Leu-Aib-Pro-Val-Aib-**Aib**-Gln-Gln-Pheol
	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Polysporins	Ac-Aib-Pro-Aib-Ala-Aib-Aib-Gln-Aib-**Leu/Ile**-Aib-Gly-**Leu/Ile**-Aib-Pro-Val-Aib-**Aib**-Gln-Gln-Pheol
	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Paracelsins	Ac-Aib-Ala-Aib-Ala-Aib-**Ala**-Gln-Aib-**Val**-Aib-Gly-Aib-Aib-Pro-Val-Aib-Aib-Gln-Gln-Pheol
Saturnisporins	Ac-Aib-Ala-Aib-Ala-Aib-**Ala**-Gln-Ala-**Leu**-Aib-Gly-Aib-Aib-Pro-Val-Aib-Iva-Gln-Gln-Pheol
Trichokonins	Ac-Aib-Ala-Aib-Ala-Aib-**Ala**-Gln-Aib-Val-Aib-Gly-Leu-Ala-Pro-Val-Aib-**Aib**-Gln-Gln-Pheol
Trichobrachins	Ac-Aib-Ala-Aib-Ala-Aib-**Ala**-Gln-Aib-**Val**-Aib-Gly-Leu-Aib-Pro-Val-Aib-Val-Gln-Gln-Pheol
Gliodeliquescins	Ac-Aib-Ala-Aib-Ala-Aib-**Ala**-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-**Aib**-Gln-Gln-Pheol
	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Trichosporins	Ac-Aib-Ala-**Ser**-Ala-**Aib**-Aib-Gln-Aib-**Leu-Aib-Gly-Leu-Aib**-Pro-Val-Aib-**Aib**-Gln-Gln-Pheol
	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Trichorzianines	Ac-Aib-Ala-Ala-Aib-**Aib**-Gln-Aib-Aib-Aib-Ser-Leu-Aib-Pro-**Leu**-Aib-**Ile**-Gln-Gln-**Trpol**
Tricholongins	Ac-Aib-Gly-Phe-Aib-Aib-Gln-Aib-Aib-Aib-Ser-Leu-Aib-Pro-Val-Aib-**Aib**-Gln-Gln-**Leuol**
Trikoningins KA	Ac-Aib-Gly-Ala-Aib-**Ile**-Gln-Aib-Aib-Aib-Ser-Leu-Aib-Pro-**Val**-Aib-**Ile**-Gln-Gln-**Leuol**
	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Trichotoxins	Ac-Aib-Gly-Aib-Leu-Aib-Gln-Aib-Aib-**Aib**-Ala-Ala-Aib-Pro-Leu-Aib-**Aib**-Gln-Valol

Table 7.3 *(Cont)*

	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Trichokindins	Ac-Aib-Ser-Ala-Aib-**Aib**-Gln-**Iva**-Leu-Aib-Ala-**Aib**-Aib-Pro-Leu-Aib-Aib-Gln-**Iol**
Trichorzins	Ac-Aib-**Gly**-Ala-Aib-**Aib**-Gln-**Aib-Val**-Aib-Gly-Leu-Aib-Pro-Leu-Aib-**Aib**-Gln-**Leuol**
	1 2 3 4 5 6 7 8 9 10 11 12 13 14
Trichovirins	Ac-Aib-**Asn**-Leu-Aib-Pro-**Ser-Val**-Aib-Pro-**Aib**-Leu-Aib-Pro-Leuol
Harzianins HA	Ac-Aib-**Asn**-Leu-Aib-Pro-**Ala-Ile**-Aib-Pro-**Iva**-Leu-Aib-Pro-Leuol
	1 2 3 4 5 6 7 8 9 10 11
Trikoningins KB	Oc-**Aib**-Gly-Val-Aib-Gly-Gly-Val-Aib-Gly-Ile-Leuol
Trichorozins	Ac-Aib-**Asn**-Ile-Leu-Aib-Pro-Ile-Leu-Aib-Pro-**Vol**
Trichogins A	Oc-Aib-Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Leuol
	1 2 3 4 5 6 7
Trichodecenins	Z-4-decenoyl-Gly-Gly-Leu-Aib-Gly-Ileu-Leuol

1. Amino acids shown in bold indicate positions where heterogeneity occurs.
2. Abbreviations for less common amino acids or derivatives: Aib, á-aminobutyric acid; Iol, isoleucinol; Iva, isovaline; Leuol, leucinol; Pheol, phenylalaninol; Trpol, tryptophanol; Vol, valinol.
3. Oc stands for octanoyl.

the residue at 18: glutamate in one is replaced by glutamine in the other (Sansom, 1993). The interest in these compounds arose from their effectiveness as antimicrobial agents towards Gram-positive organisms. Since these early studies, a remarkable number of similar compounds have been isolated and identified mainly from *Trichoderma* species although they occur in most species and strains of *Trichoderma* and *Gliocladium* (Brückner *et al.,* 1989). However, all of these occur as microheterogeneous mixtures of closely related polypeptides, differing only in the type of amino acids at one or more positions, a reflection of the non-ribosomal mechanism of biosynthesis. Consequently, only one example of each of the different types is given and the position(s) where heterogeneity occurs is indicated by showing the amino acid in bold font (Table 7.3).

Different sub-classes of peptaibols are recognized: the long sequence peptaibols (18–20 residues), short sequence peptaibols (11–16 residues) and liptopeptaibols which contain 7 or 11 residues with the N-terminal amino acid acylated by a short lipidic chain instead of an acetyl group.

Apart from the alamethicins, peptaibols containing 20 amino acid residues are the suzukacillins from *T. viride* (Katz *et al.,* 1985), the polysporins from *T. polysporum* (New *et al.,* 1996), the paracelsins from *T. reesei* (Brückner *et al.,* 1984) and *T. saturnisporum* Hammill (Ritieni *et al.,* 1995), the saturnisporins from *T. saturnisporum* (Rebuffat *et al.,* 1993; Ritieni *et al.,* 1995), trichosporins from *T. polysporum* (Link: Fr.) Rifai (Iida *et al.,* 1993) and *T. koningii* (Huang *et al.,* 1995b), trichokonins from *T. koningii* (Huang *et al.,* 1995b), the trichobrachins from *T. longibrachiatum* (Brückner *et al.,* 1990), and the gliodeliquescins from *G. deliquescens* (Brückner and Przybylski, 1984) and *T. koningii* (Huang *et al.,* 1995b).

A group of peptaibols containing 19 amino acid residues are also known: the trichorzianines from *T. harzianum* (Bodo *et al.,* 1985; El Hajji *et al.,* 1987), the tricholongins from *T. longibrachiatum* (Rebuffat *et al.,* 1991), and the trikoningins KA from *T. koningii* (Auvin-Guette *et al.,* 1993). The first set of compounds containing 18 amino acid residues were the trichotoxins from *T. viride* (Brückner *et al.,* 1985). More recently, the structures of the trichokindins from *T. harzianum* (Iida *et al.,* 1994) and the trichorzins from *T. harzianum* (Hlimi *et al.,* 1995) have been established.

Short-sequence peptaibols are represented by the trichovirins from *T. viride* (Brückner and Koza, 1992), the harzianins HA (14 residues) from *T. harzianum* (Rebuffat *et al.,* 1995), the trikoningins KB (11 residues) from *T. koningii* (Auvin-Guette *et al.,* 1993), the trichorozins from *T. harzianum* (Iida *et al.,* 1995) and the trichorovins from the conidia of *T. viride* (Wada *et al.,* 1995, 1996). The trichogins A from *T. longibrachiatum* (Auvin-Guette *et al.,* 1992) and the trichodecenins from *T. viride* (Fujita *et al.,* 1994) are examples of lipopeptaibols. A rather unique set of peptaibols are the trichopolyns from *T. polysporum* **(116, 117)** (Figure 7.8). They are characterised by the presence of an *R*-2-methyldecanoyl group esterifying the N-terminal amino acid, a 2-amino-6-hydroxy-4-methyl-8-oxodecanoic acid residue at position 2 and the unusual C-terminal group (Fujita *et al.,* 1981; Mihara *et al.,* 1994).

7.3.10 Unclassified metabolites

The production of isoamyl alcohol, octan-3-one, 1-octen-3-ol and octanol from cultures of *Trichoderma* has been established (Saito *et al.,* 1979). The hydroxy acid **(118)**

116 R_1 = Ile; R_2 = (R)-2-methyldecanoyl-Pro-

117 R_1 = Val; R_2 = (R)-2-methyldecanoyl-Pro-

Figure 7.8 Structures of trichopolyns I and II.

(Figure 7.9), isolated from cultures of *G. deliquescens,* almost certainly reflects a fragment of the side chain of a sterol involved in the production of the viridins (see Figure 7.7). Uracil **(119)** has been isolated from cultures of *T. harzianum* (Huang *et al.,* 1995a). A compound that has been assigned the oxazole structure **(120)** has been identified, independently, in cultures of *T. harzianum* (Lee *et al.,* 1995a) and an unidentified *Trichoderma* species (Hashimoto *et al.,* 1995). The lack of coincidence of physico-chemical properties between the two compounds suggests either that one or both the samples are impure or that they are diastereomeric. The sample of Hashimoto *et al.* (1995) was found to inhibit melanin synthesis in larval *Bombyx mori*. The ceramide **(121)**, isolated from *T. harzianum* grown on a yeast-malt culture medium (Huang *et al.,* 1995a), has previously been reported as a constituent of ceramide mixtures in bran and endosperm of rice grains and in the leafy stems of rice. The ceramide **(121)** probably arises from the combination of the amino acid threonine with palmitoyl CoA (C16) and subsequent amide formation with a C24 fatty acyl CoA. Ceramides are constituents of the lipid portion of cell membranes and membranes of cell organelles. Valinotricin **(122)** (Fujita *et al.,* 1984) is an ester that can formally be considered to be generated by condensation of N-formyl valine and N-formyl valinol.

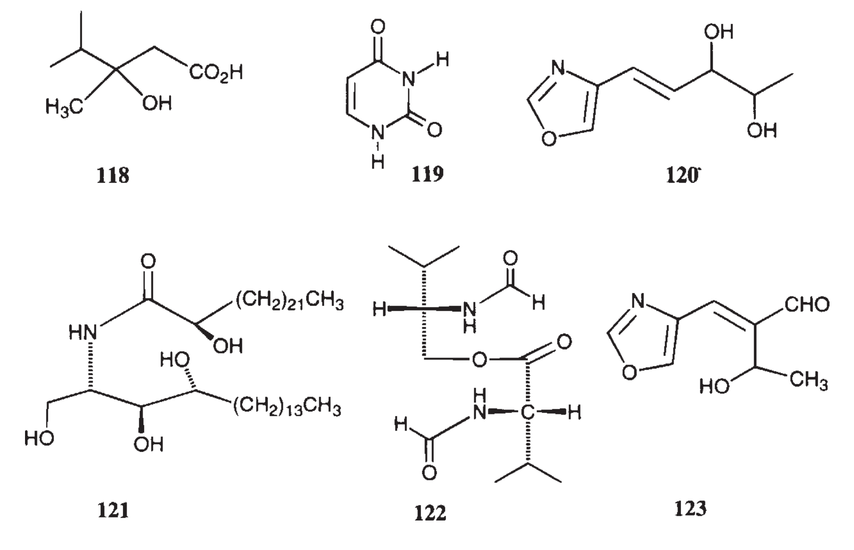


Figure 7.9 Unclassified metabolites.

7.3.11 Addendum

Another oxazole **(123)** that inhibits the biosynthesis of melanin was isolated from a *Trichoderma* sp. (Takeahashi *et al.,* 1996). Two new metabolites which inhibit the binding of the regulation of virion expression protein (REV) to the REV-responsive element in HIV infection have been isolated from *T. harzianum* (Qian-Cutrone *et al.,* 1996). The structures of harziphilone **(124)** and fleephilone **(125)** suggest that they are related to the azaphilones, well-known polyketide metabolites (Turner and Aldridge, 1983).

7.4 Correlation of secondary metabolites

From this survey of the secondary metabolites produced by *Trichoderma* and *Gliocladium* species, a few trends can be discerned. We emphasise that these correlations are made by considering the original names allocated to source organisms as given in the primary literature and listed in Table 7.1. Reclassifications of species, e.g. *G. virens* to *T. virens,* have been ignored.

The secondary metabolism of these two genera is dominated by compounds derived from the polyketide pathway **(18–55)** and from elaboration of amino acid metabolism (**81–117** and the peptaibols). Whereas sesquiterpenes **(56–68)** and sterolderived metabolites **(71, 72, 74–79)** are well represented, the monoterpenes, diterpenes **(60)** and triterpenes **(70, 73, 80)** do not seem to be as common. A comparison of the metabolites commonly found in *Trichoderma* with those found in *Gliocladium* also reveals some interesting facts. So far, metabolites derived from the TCA cycle are restricted to *Trichoderma* strains. Within the polyketides, *Trichoderma* metabolites arise from pentaketides and larger intermediates and *G. roseum* appears to specialise in tetraketide-derived metabolites. The only case of a tetraketide metabolite from a *Trichoderma* species is harzianopyridone **(40)** whose biosynthesis involves an amino acid. The trichothecene sesquiterpenes seem to be restricted to *Trichoderma* whereas the viridin metabolites are characteristic of *Gliocladium* species. This suggests that the *T. viride* strain examined by Golder and Watson (1980) was indeed a *"Gliocladium"* species and that the isolate of *T. koningii* reported by Berestetskii *et al.* (1976) may also have been a *"Gliocladium"*. Generally, the isocyano metabolites are expressed by *Trichoderma* spp. and the gliotoxin series by *"Gliocladium"* species; the strain of *T. lignorum* used by Weindling (1934) was in fact *Gliocladium (Trichoderma) virens* and it seems likely that the *T. hamatum* strain examined by Hussain *et al.* (1975) was in fact a *"Gliocladium"* strain. The metabolic profile of *T. virens,* unlike the other characteristics studied (Volume 1, Chapter 1), supports the retention of *T. virens* within the genus *Gliocladium*. As mentioned previously, the peptaibols are characteristic of both *Trichoderma* and *Gliocladium,* although most studies have concentrated on species of the former.

7.5 Biological activity of secondary metabolites

The biological activity of some of the secondary metabolites from *Trichoderma* and *Gliocladium* has been alluded to previously and a complete list is given in

Table 7.2. The main interest is in those compounds that exhibit antibiotic activity since they are more likely to be implicated in the effectiveness of the strain producing them as a biological control agent. The synergistic effect with enzymes produced by them also appears to be important (Belanger *et al.,* 1995; Di Pietro *et al.,* 1993; Lorito *et al.,* 1994; Schirmbock *et al.,* 1994). Whilst it is obvious that these species are prolific producers of secondary metabolites, very little is known about the factors that determine the production of individual metabolites. In this review, the implication has been that strains producing different metabolites are themselves different. However, conidial progenies of field isolates have been shown to vary in their abilities to produce 6PP (Worasatit *et al.,* 1994). Moreover, there are tentative indications that metabolite production may depend, in a qualitative and quantitative manner, on the medium on which a particular strain is cultured (Brian and Hemming, 1945; Hanssen and Urbasch, 1990; Jackson *et al.,* 1991; Lumsden *et al.,* 1992a; Wright, 1956; Yong *et al.,* 1985) as well as on the age of the culture (Ghisalberti and Sivasithamparam, 1991). The observation that relative simple mutations can activate otherwise "silent" biosynthetic pathways and that environmental conditions may favour one or more of a number of possible pathways is not unexpected. In this context, recent results with trichodiene synthase are worth considering. This enzyme, isolated from various fungal sources, catalyses the conversion of farnesyl pyrophosphate to trichodiene, the parent hydrocarbon of the trichotecane family of antibiotics and mycotoxins (e.g. **65–68**). Site-directed mutagenesis of the synthase yielded mutants which converted farnesyl diphosphate not only to trichodiene but also to other sesquiterpene hydrocarbons (Cane and Xue, 1996). The structure of the new hydrocarbons could be rationalised as arising from intermediates which are precursors of trichodiene. These results suggest that alteration of the residues at the active site of the enzyme results in a small but important alteration in the precise positioning and folding of the substrate thus allowing "aberrant" cyclization products.

In examining the structures of the different metabolites exhibiting antibiotic activity, one can distinguish two main types. The simple aromatic compounds **(6–19)**, the pyrones **(38, 39, 45–48)**, the butenolides **(41, 42)** and the isocyano metabolites **(81–99)** are low molecular weight, relatively non-polar substances that have a significant vapour pressure. The production of these in the soil environment would be expected to result in high local concentrations of antibiotics that may exert their influence over a "distance". However, members of this group may diffuse through water and also exert effects a small distance from the producing hyphae. On the other hand, high molecular weight, polar metabolites may best express their activity on contact interactions between *Trichoderma* and *Gliocladium* species and their antagonists. Of particular relevance to this type of interaction are the peptaibols. As amphiphilic compounds, they would be expected to exhibit "detergent-like" properties. They have been shown to perturb the permeability properties of phospholipid bilayers and to exert antibiotic activity against Gram-positive and Gram-negative bacteria. The structural properties of peptaibols impart an amphiphilic helix-favouring character which allows these peptides to aggregate in a membrane and form ion channels (Iida *et al.,* 1995). These modifications of the membrane structures lead to uncoupling of oxidative phosphorylation, release of catecholamines from adrenal chromaffin cells, inhibition of amoeba cell multiplication and, at high concentrations, cell lysis (Huang *et al.,* 1995b). Gliodeliquescin A is a potent activator of the L-type Ca^{2+} channel in cardiac membranes (Huang *et al.,* 1995b).

7.6 Concluding remarks

Apart from the considerable number of peptaibols, over 120 medium molecular weight compounds have been identified from *Trichoderma* and *Gliocladium* species. The activity in this field over the last decade is remarkable and the pace continues unabated at the present time. The application of RAPD methods in the correlation of morphological and cultural properties and metabolic profiles appears to hold some promise for the characterization of individual strains (Fujimori and Okuda, 1994). For most cases, a correlation between production of antibiotics by an isolate and its effectiveness as a biological control agent is still a matter of conjecture, although molecular biology techniques may provide means of establishing a valid correlation. Of some significance also is the study of the metabolites from these species as sources of compounds with biological activities apart from those associated with antibiosis. These approaches will ensure that interest in *Trichoderma* and *Gliocladium* species will continue for some considerable time.

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The safety of *Trichoderma* and *Gliocladium*

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8.1 Introduction

The safety assessment of a microorganism is a multifaceted task that involves thoroughly reviewing the history and properties of the organism of interest, testing the products manufactured for the presence of any harmful metabolites, and validation or accreditation of the production process. The guidelines and recommendations for safe use of microbial products in applications such as food and feed processing or biocontrol may vary in different countries. However, the tests and the methods used in a safety evaluation of, for example, enzyme products are in general based on the guidelines formulated by recognised authorities such as the Organisation for Economic Cooperation and Development (OECD), Joint FAO/WHO Expert Committee on Food Additives (JECFA), European Economic Community (EEC) and the Food and Drug Administration of the USA (FDA). Regulations concerning genetically modified microorganisms are documented in international agreements, directives and recommendations, EC directives and decrees and, at the national level, by legislation or guidelines concerning such aspects as the labelling of recombinant products.

Both mutant and recombinant *Trichoderma reesei* strains are currently used in the biotechnology industry for the production of hydrolytic enzymes (reviewed in Volume 2, Part 3). Other species of *Trichoderma* and representatives of the genus *Gliocladium* are being intensively studied as microbial antagonists for the control of plant pathogenic fungi (reviewed by Chet, 1987; Lewis *et al.,* 1995; Lorito *et al.,* 1994; Papavizas, 1985; and papers in Volume 2, Part 2). The commercial use of *T. reesei* has necessitated the close examination of this organism with respect to worker safety and product use (reviewed in Nevalainen *et al.,* 1994). When *Trichoderma* and *Gliocladium* are considered for use as biological control agents, the impact on the environment of their release has to be assessed in addition to potential hazards to workers involved in product manufacture.

8.2 Natural environments and functions of *Trichoderma* and *Gliocladium* in the ecosystem

Trichoderma and *Gliocladium* are common soil hyphomycetes in all climate zones (Domsch *et al.,* 1980), ranging from Antarctica (Corte and Gestro, 1992; R.Seppelt,

Table 8.1 Occurrence of *Trichoderma* and *Gliocladium* in agricultural and non-soil environments

Environment of isolation	Genus	Reference
Coffee pulp	*Trichoderma*	Roussos *et al.* (1995)
Pistachio nuts	*Trichoderma*	Bilgrami and Ghaffar (1994)
Freight containers	*Trichoderma*	Hill *et al.* (1995)
Hard coal	*Trichoderma*	Bublitz *et al.* (1994)
Historical monuments	*Trichoderma*	Gomezalarcon *et al.* (1995)
Leather goods and shoes	*Trichoderma*	Birbir *et al.* (1996)
TNT-contaminated composts	*Trichoderma*	Bennett *et al.* (1995)
Sour cherry orchards	*Trichoderma*	Olszak (1994)
	Gliocladium	
Dry beans	*Gliocladium*	Tseng *et al.* (1995)
Feed stuff	*Gliocladium*	Atalla and El Din (1993)

personal communication) to the tropics. As saprophytic organisms, they can use a wide range of compounds as carbon and nitrogen sources and can secrete a variety of enzymes such as cellulases and hemicellulases to break down recalcitrant plant polymers into simple sugars for energy and growth. Examples of different natural and geographical environments where *Trichoderma* spp. have been isolated comprise mixed hardwood forests, garden soil, micromycete complex in pea rhizosphere (Polyanskaya *et al.,* 1994), mangrove estuaries in Hong Kong (Sadaba *et al.,* 1995), rice fields in Bangladesh (Kamal and Shahjahan, 1995), Dutch coastal foredunes (De Rooij van der Goes *et al.,* 1995) and the Ipanema beach in Rio de Janeiro (Sarquis and De Oliveira, 1996). Both *T. pseudokoningii* and *G. (T.) virens* have been isolated from calcixeroll soil in Northern Greece under macchia vegetation (Vardavakis, 1990).

Interestingly, there are also reports on the colonisation of cysts or females of the soybean pathogen, cyst nematode *Heterodera glycines,* by *Gliocladium* sp. in the United States (Chen *et al.,* 1994) and of *H. glycines* eggs isolated from soil in Brazil (Silva *et al.,* 1994). *T. harzianum, T. koningii* and *G.(T.) virens* have been found to be effective in the biological control of the nematode *Meloidogyne javanica* on tomato and okra (Parveen *et al.,* 1993). Grazing of soil arthropods on hyphae of conidial fungi such as *T. harzianum* and dispersal of fungal conidia by insects may also contribute to the biology and fertility of the soil (Klironomos and Kendrick, 1996; Vega *et al.,* 1995). Because of their degradative properties, *Trichoderma* and *Gliocladium* are also found growing in the various non-soil environments presented in Table 8.1.

Soil is a crowded competitive environment where production of cell wall digesting enzymes and antifungal or antibacterial substances can promote survival and colonisation by antibiosis and mycoparasitism. In addition to enzymes, *Trichoderma* and *Gliocladium* spp. are also producers of various metabolites with toxic or antibiotic properties (Brückner *et al.,* 1990; Dennis and Webster, 1971; Ghisalberti and Sivasithamparam, 1991; Huang *et al.,* 1995; Iida *et al.,* 1995; Parker *et al.,* 1995; Rebuffat *et al.,* 1995; Stipanovic and Howell, 1982; Tomoda *et al.,* 1992; Wada *et al.,* 1995) (see also Volume 1, Chapter 7 and Volume 2, Chapter 8). Production and the mode of action of antifungal substances and host cell wall digesting enzymes are discussed in detail in papers in Volume 2, Chapters 4, 5 and 8.

8.3 Safety issues related to product manufacture and use

The two main groups of products from *Trichoderma* and *Gliocladium* are (a) extracellular enzymes produced industrially in liquid culture and sold free of fungal biomass and (b) biological control preparations consisting of fungal spores and mycelium. Aspects requiring consideration when assessing the safety of a biological product are possible allergenicity, pathogenicity and toxicity of the organism employed and any associated threat to factory worker, end-user or to the general public following accidental or deliberate release into the environment. A thorough knowledge of the properties of the organism is thus essential. In addition, an evaluation should be carried out on the safety of the product itself. Typically, full safety evaluations of the production organism and the product are conducted on test animals. These consist of studies on oral toxicity, acute inhalation toxicity, skin and eye irritation, skin sensitization, dietary toxicity and mutagenic evaluation. In addition, the organism is tested for pathogenicity and for the production of antibiotics and toxins. The execution and selection of tests may be based on the guidelines of the OECD (1984) and EEC (1992, 1994), and on recommendations by JECFA (1981, 1990, 1992) concerning the use of food and feed additives.

It is desirable that a production process comply with the ISO 9002 standards. In the case of fermentation, whether based on solid or liquid substrate technology, this should encompass the entire process, with the inclusion of both upstream and downstream processing as well as product formulation. When approval is sought for the manufacture and sale of an enzyme as a food additive in the USA, a well-documented application (Food Additive Petition) may be required by the FDA. In this case, marketing of the product is pursuant upon the publication of conditions and terms of FDA approval, if granted. Alternatively, a product may be marketed when granted a GRAS (Generally Recognized As Safe) status by the FDA or following self-affirmation as GRAS by the manufacturing company. In regulatory documentation, enzymes are mentioned as food and feed additives, or processing aids or are discussed under chemical substances acts. According to AMFEP (Association of Microbial Food Enzyme Producers), the evaluation of all enzyme products should be based on their particular properties and not the technology applied for the development of the production organism. All enzyme products, irrespective of how they have been produced, can be marketed only after their safety has been confirmed according to international standards and approval procedures. In many cases, enzymes function as process biocatalysts without a specific role in the final product and should thus be treated as processing aids (AMFEP, 1992).

With regard to products used in biological pest and phytopathogen control, registration requirements are generally less stringent than those applying to the use of chemicals in similar applications (Holdom *et al.,* 1996; Pitt, 1995; Taverner, 1995). Non-transgenic biocontrol organisms are frequently ubiquitous in nature and their introduction into the environment has to be assessed in terms of either a transient or an enduring increase in the natural levels of the organisms concerned. In this respect they can be considered to fall into a similar category to organisms used as soil inoculants or biofertilisers.

Following the 1986 OECD report, *Recombinant DNA Safety Considerations* (referred to colloquially as the “Blue Book”), a working group (GNE or Group of National Experts on Safety in Biotechnology) of this organisation was convened in 1988 to further develop and refine the principles for safety in biotechnology.

Although their debate was mainly directed at the products of molecular biology, the safety principles published in the volume *Safety Considerations for Biotechnology* (1992) have application to the unnatural introduction of any organism into the environment. In this latter report, the GILSP (Good Industrial Large Scale Practice) criteria proposed in the 1986 report are expanded and attention is also given to good development principles (GDP) for small-scale field trials. Of particular interest to workers involved in the soil application of *Trichoderma* and *Gliocladium* as biological control agents is the second in the series of special reports published by the GNE of the OECD entitled *Safety Considerations for Biotechnology: Scale-up of Micro-organisms as Biofertilisers* (1995). This report stresses the importance of familiarity with the organism, with the environment into which the organism is introduced and with any introduced traits. An attempt should be made to establish or predict the interactions between the organism and the environment which may result from the release of the organism. Knowledge of this type may be derived from the literature or accumulated through practical experience, laboratory research, greenhouse trials, and small-scale field trials. In Table 8.2, the specific areas of knowledge that may be of use are summarised. The information can identify potential hazards related to the use of the biological agent and can allow appropriate hazard management strategies to be formulated for application during scale-up.

8.3.1 Pathogenicity, toxicity and allergenicity

Trichoderma and *Gliocladium* have not been considered as pathogens for humans in general. However, a number of saprophytic fungi have been reported to cause infection under exceptional conditions, especially in immunocompromised patients (Anaissie *et al.,* 1989; Bodey, 1988; Vartivarian *et al.,* 1993). So far seven cases of *Trichoderma* infection have been reported in humans. *T. viride* (Loeppky *et al.,* 1983) and *T. koningii* (Ragnaud *et al.,* 1984) were identified as causal agents for peritonitis in patients with chronic renal failure and continuous ambulatory peritoneal dialysis. *T. viride* has also been found in a liver transplant recipient (Jacobs *et al.,* 1992) and two immunocompromised patients, one of whom succumbed to pulmonary *Trichoderma* infection resembling aspergillosis (Escudero Gil *et al.,* 1976). The other, who inadvertently received contaminated intravenous infusion fluid, responded to treatment with amphotericin B (Robertson, 1970). In a bone marrow transplant recipient, *T. pseudokoningii* was the cause of a fatal infection (Gautheret *et al.,* 1995). Recently, a brain abscess in a leukaemia patient due to *T. longibrachiatum* infection was cured by surgical removal of the abscess and prolonged antifungal therapy (Seguin *et al.,* 1995). In this instance, molecular techniques such as ITS-1 sequencing within rDNA and RAPD analysis of genomic DNA were applied to obtain rapid and accurate identification of the causative organism.

A likely route for the contraction of the fungal infection leading to the brain abscess described above was environmental inhalation followed by the contiguous spread of infection through the sinuses (Seguin *et al.,* 1995). Presence of bacterial or fungal spores in indoor air may also be associated with respiratory allergies. In a study of fungal contamination of indoor air in homes of patients with asthma bronchiale, *Trichoderma* spp. were found in low amounts but were not among major causes of allergy (Senkpiel *et al.,* 1996). Among species of *Trichoderma,* allergenic properties of *T. viride* are best documented. In the home, *T. viride* is commonly

Table 8.2 Areas of familiarity useful in the assessment of the risk/safety associated with the scale-up of microorganisms to be used in the control of plant disease

Area of familiarity	Points for consideration
Microorganism	Understanding of the taxonomic status of organism Historical information on use of same or related microorganism Natural occurrence of the same or related microorganism Physical, climatic and nutritional factors affecting occurrence and proliferation of microorganism
Familiarity with microbe–pathogen interaction	Studies on nature of antibiotic agent or mode of parasiticity Studies on the relationship between physical and nutritional cultural parameters and parasiticity or production of antibiotic Potential for development of resistance by the host pathogen Potential for use in combination with other agents
Familiarity with a trait introduced by recombinant gene technology (functional or regulatory genes or marker genes introduced to protect intellectual property or to track residual activity)	Knowledge of phenotypic traits new to the organisms concerned may be gained from the following: • the species of origin • knowledge of the genetic insert including regulatory sequences • experience with the same trait in other organisms
Familiarity with the environment	Effect of increase in population of a naturally-occurring organism, including the following: • potential for the harming of beneficial organisms • potential for damage to wild fauna and flora • potential for harm to crops and stock • potential for harm to human population • effect on the above of increased presence in water resources • effect of disposal of waste product from manufacturing processes

found on tapestry, in moist areas and in kitchens on biological garbage and under unglazed ceramics. Inhaled *Trichoderma* spores have been connected to a form of allergy called woodworkers disease (Kabi Pharmacia Diagnostics AB, 1992).

Results of tests conducted with animals indicate that, as is the case with humans, *Trichoderma* can only act as a pathogen under abnormal conditions (Hjortkjaer *et al.,* 1986). A pathogenicity study carried out by these workers showed that while *T. reesei* could not be considered pathogenic to healthy mice, guinea pigs or rabbits, pathogenicity could be demonstrated when a large inoculum was given to immuno-suppressed mice. However, the occurrence of a *Trichoderma* infection in nature has been reported by Foreyt and Leathers (1985) who isolated this organism

from an alligator. Species of *Trichoderma* such as *T. koningii, T. viride* and *T. harzianum* produce a variety of antibiotic antifungal peptides called peptaibols that are capable of interacting with cell membranes (for references, see Rebuffat *et al.,* 1995 and Volume 1, Chapter 7). Studies on peptaibols, related to transmembrane channel forming activity, have demonstrated their ability to uncouple oxidative phosphorylation in rat liver mitochondria (Okuda *et al.,* 1994), enhance catecholamine secretion in bovine adrenal chromaffin cells (Tachikawa *et al.,* 1991), inhibit amoeba cell multiplication (El Hajji *et al.,* 1989) and act as channel agonists in bullfrog cardiac myocytes (Huang *et al.,* 1994). Because of these demonstrated effects on mammalian cells and the fact that at high concentrations peptaibols can cause cell lysis, care should be taken in handling large amounts of fungal product.

T. virens is known to produce potent toxins such as viridin and gliotoxin (discussed in Volume 1, Chapter 7). Gliotoxin, an epidithiodiketopiperazine, has an LD50 of 25–50 mg per kg, as demonstrated in rats and mice by Taylor (1971). Waring *et al.* (1995) noted the inactivation of horse liver dehydrogenase by this toxin. A property that has aroused considerable interest is the ability of this metabolite to selectively suppress the immune system, indicating a potential for use as an immunomodulating agent in organ transplants (McMinn *et al.,* 1991; Pahl *et al.,* 1996; Sutton *et al.,* 1995). This immunosuppressive capacity suggests a direct role for gliotoxin in the pathology of fungus-associated respiratory infection or aspergillosis (Pahl *et al.,* 1996).

8.3.2 **Trichoderma reesei** ***enzyme products***

High cellulase-producing mutants of *Trichoderma reesei* have been employed as enzyme producers for the biotechnology industry for over 20 years. Mutants developed by industry from the wild-type isolate QM6a (Mandels and Reese, 1957) have accumulated a long track record of safe use in industrial-scale enzyme production and application in the food, animal feed and pharmaceutical industries (reviewed by Nevalainen *et al.,* 1994). Further support for the safety of this organism is provided by the absence of any evidence indicating pathogenicity to healthy man or animals. *T. reesei* has been recognised as safe in the AMFEP list of sources for microbial food enzymes (AMFEP, 1992).

Considerable experience in the safe use of genetically modified *Trichoderma reesei* strains in industrial-scale processes has been accumulated over the years, indicating that this fungus can serve as a safe host for a variety of harmless gene products. *T. reesei* strains producing cellulases have been evaluated as belonging to Group I (low risk level) microorganisms (EC Directive 90/219/EEC). Pathogenicity tests carried out with genetically engineered *T. reesei* producing modified enzyme profiles, e.g. lacking the main cellobiohydrolase, have shown that modification does not alter their safety status (Huuskonen, 1990).

Proteins in general can trigger allergic responses, either by inhalation or exposure to skin or eyes. The results of inhalation, irritation and sensitization tests of a commercial *T. reesei* cellulase enzyme preparation, Celluclast™, indicated that occupational health precautions generally taken in the manufacture and use of the enzyme preparations are adequate (Hjortkjaer *et al.,* 1986). With another cellulase product, Barlican™, a strong skin-sensitizing potential in the sensitive guinea pig model argued for somewhat more stringent occupational health precautions in the

manufacture and use of this enzyme preparation (Coenen *et al.,* 1995). Industrial enzyme preparations are routinely tested for the absence of antibiotics and mycotoxins in detectable amounts. In several European countries, *T. reesei* cellulase preparations have been approved for use in food and animal feed by the relevant authorities.

8.3.3 Trichoderma *and* Gliocladium *biocontrol preparations*

Research and development of *Trichoderma* and *Gliocladium* isolates as biological agents for the control of fungal phytopathogens is discussed in Volume 2, Part 2; Chapter 11 discusses many of the specific products mentioned below. *Trichoderma harzianum* preparations have only recently entered the market as agents for the control of fungal plant diseases. A product based on *T. harzianum* strain T39 and sold under the brand-name Trichodex™ 25P is marketed for the control of grey mould *(Botrytis cinerea)* on grapevines and sundry vegetable crops in several countries (O'Neill *et al.,* 1996). Integrated application of fungicides with the biocontrol agent achieved a suppression of the growth of the grey mould as good as that obtained with the chemicals alone and a more consistent control than that by the biocontrol agent alone (Elad *et al.,* 1994). While this strain was not particularly effective in the control of black scurf disease in potatoes caused by *Rhizoctonia solani* (Wicks *et al.,* 1995), researchers in Zimbabwe found one of their isolates, *T. harzianum* T77*,* to be very efficient in the control of sore shin disease in tobacco (Cole and Zvenyika, 1988). This disease is caused by a complex combination of fungi of which the main components are *Rhizoctonia solani* and *Fusarium solani.* Cole has also demonstrated a reduction in the severity of black shank *(Phytophthora parasitica)* by this preparation (J.Galloway, personal communication). A product based on strain T77 is currently in commercial production in Zimbabwe with production figures of 131 tonnes recorded for 1995 (J.Galloway, personal communication). According to Wicks *et al.* (1995), a commercial preparation of *T. koningii* (J.B.Biotech Inc., Ventura, CA, USA) is widely promoted as a soil additive for the control of a broad range of soil-borne pathogens in South Australia. A *T. virens*-based agent marketed as Gliogard™ was the first fungal biocontrol agent to be awarded US Environmental Protection Agency (EPA) approval and is recommended for the control of *R. solani* and *Pythium ultimum,* the causal agents of the damping-off of ornamentals (Lumsden and Locke, 1989), and of southern blight of carrot and pepper, caused by *Sclerotium rolfsii* (Ristaino *et al.,* 1994). *T. viride* and *T. polysporum* have been incorporated into products sold in France and the United Kingdom for the control of silver leaf disease on trees and verticillium wilt in mushrooms (Ricard, 1981). A series of products marketed as Planter Box T-22™, Root Shield™, Bio-Trek™, and other names are produced and marketed primarily in the USA for control of diseases of agronomic and row crops, greenhouse crops, and golf course turf, respectively (Volume 2, Chapter 11). The continued use of these products associated with monitoring of environmental impact will, in time, provide a historical record with increasing validity for use in the assessment of safety risk.

8.3.4 Potential novel uses

Recently, two new inhibitors of *c-fos* proto-oncogene induction have been isolated and characterised from a fermentation broth of *Gliocladium* spp. Indications are

that these diketopiperazines could serve as therapeutic agents for the control of neo-plastic disease (Chu *et al.,* 1995). Novel glisoprenins, isolated from culture broth of *Gliocladium* spp. as colourless oils, have shown to act as inhibitors of acetyl-CoA: cholesterol acyltransferase enzyme inhibiting cholesterol absorption in a hamster model (Tomoda *et al.,* 1992). Anthrotainin, a new tetracyclic compound with the ability to act as an antagonist to the neuromodulating Substance P, has been characterised from a culture of *Gliocladium catenulatum* (Wong *et al.,* 1993). Should one or more of these products be used in therapeutic applications, the safety principles outlined above will have to be expanded.

8.4 Conclusion and recommendations

The saprophytic soil fungi *Trichoderma* and *Gliocladium* can be considered non-pathogenic for a healthy human under normal exposure to these organisms. During the manufacture of enzyme or biocontrol products in liquid fermentation, the number of spores released into the environment is generally insignificant so that the development of an allergy is unlikely. However, the level of aerial contamination should be monitored closely and the workers submitted to routine medical examinations. Since inhalation of enzyme-containing dust or aerosols and skin and eye contact with enzyme powder can also cause allergic reactions, the level of factory pollution by these substances should be kept to a minimum and necessary precautions taken to ensure protection of the health of the employees. Where solid substrates are used in open trays, the risk of contamination increases dramatically. The process should be designed to achieve maximum containment of the product and adequate occupational safety precautions should be enforced.

Apart from the above considerations, there appears to be very little evidence for concern regarding the application of *Trichoderma reesei* in the commercial manufacture of enzymes or for the use of the enzyme products in the feed and food industries. In view of the relatively recent and in other cases impending commercialisation of *Trichoderma* and *Gliocladium* species as biocontrol agents, monitoring of the effects of the release of these organisms into the environment should be undertaken on a continuing basis with a view to the accumulation of evidence for safety/risk assessment. Pathogenicity tests conducted to date do not suggest that these organisms pose a threat to human or animal health. Aspects such as the impact on indigenous plant and microbial populations, persistence in the soil, dispersal to areas removed from the site of application and development of resistance by the target organism may need further investigation under field conditions.

Trichoderma and *Gliocladium* are also potential sources for novel metabolites. The discovery of novel compounds and the advent of hybrid and recombinant biocontrol strains emphasizes the necessity for continuous monitoring, and revision of guidelines and practices for safe application of fungal products.

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PART TWO

Molecular biology and genetics

Trichoderma spp. genome and gene structure

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9.1 Introduction

Fungi in the genus *Trichoderma* have been used for the production of lytic enzymes and to control a wide range of plant pathogenic fungi. In the last years, much progress has been made in elucidating the molecular biology of *Trichoderma* spp. The objective of this chapter is to provide a summary of data concerning size and organization of the genome and gene structure in *Trichoderma* spp., mainly translation control sequences and codon usage. This summary will aid in the investigation of the molecular genetics of this genus.

9.2 Size and organization of the genome

Filamentous fungi usually contain haploid nuclei and a relatively small genome, frequently about 25 to 50 Mb (for a review, see Skinner *et al.,* 1991). The development of pulse-field gel electrophoresis (PFGE) has allowed electrophoretic karyotyping of several yeasts and filamentous fungi. The use of PFGE and molecular karyotyping technology has led to the assignment of cloned genes to chromosomal locations. New understandings can arise through the utilization of this technology; for example, molecular karyotyping can aid in the detection of translocations and variations in chromosome number and can be used to generate chromosome-specific sublibraries. Chromosomal DNA from *Trichoderma* spp. has been separated by using different PFGE techniques, e.g. contour-clamped homogeneous electric field, rotary electrode, and transverse-alternating field electrophoreses (Gilly and Sands, 1991; Hayes *et al.,* 1993; Herrera-Estrella *et al.,* 1993; Mäntylä *et al.,* 1992). The estimated genome sizes and chromosome numbers of *Trichoderma* spp. range from 31 to 39 Mb and from 3 to 7, respectively (see Volume 1, Chapter 11). Chromosomes differed substantially in size. The sizes of the individual chromosomes indicate significant variation between the cellulolytic *T. reesei* and those

Table 9.1 *Trichoderma* spp. gene sequence database

Gene[a]	Product	Organism	Reference	Accession[b]
hfb1	hydrophobin I	*T. reesei*	Nakari-Setela *et al.* (1996)	Z68124
cre1	CREI protein	*T. harzianum*	Ilmen *et al.* (1996)	X95369
pkc1	protein kinase C	*T. reesei*	Morawetz *et al.* (1996)	U10016
tef1	translocation elongation factor 1α	*T. reesei*	Nakari *et al.* (1993)	Z23012
cbh II	cellobiohydrolase II	*T. reesei*	Chen *et al.* (1987)	M55080
prb1	alkaline proteinase	*T. harzianum*	Geremia *et al.* (1993)	M87518 and M87519
pgk1	3-phosphoglycerate kinase	*T. reesei*	Vanhanen *et al.* (1991)	M81623 and M61878
bg11	β-D-glucoside glucohydrolase	*T. reesei*	Mach (1994)	U09580
ech-42	chitinase	*T. harzianum*	Hayes *et al.* (1994); Carsolio *et al.* (1994)	X79381
cbh1	cellulose 1,4-β-cellobiosidase	*T. koningii*	Wey *et al.* (1994)	X69976
tub1	beta-tubulin	*T. viride*	Goldman *et al.* (1993)	Z15054
tub2	beta-tubulin	*T. viride*	Goldman *et al.* (1993)	Z15055
egIII	endoglucanase III	*T. reesei*	Saloheimo *et al.* (1988)	M19373
eg15	endo-1-4-β-glucanase V	*T. reesei*	Saloheimo *et al.* (1994)	Z33381

Table 9.1 *(Cont)*

Gene[a]	Product	Organism	Reference	Accession[b]
eg11	cellulase	*T. longibrachiatum*	Perez-Gonzalez (unpublished)	X60652
pk11	pyruvate kinase	*T. reesei*	Schindler *et al.* (1993)	L07060
chit33	chitinase	*T. harzianum*	Limon *et al.* (1995)	X80006
pyr4	orotidine-5′-phosphate decarboxylase	*T. harzianum*	Heidenreich and Kubicek (1994)	U05192
axe1	acetyl xylan esterase	*T. reesei*	Margolles-Clark *et al.* (1996b)	Z69256
18s rRNA	18S rRNA	*T. harzianum*	Schlick *et al.* (1994)	Z48812
5.8s rRna	5.8S rRNA	*T. harzianum*	Schlick *et al.* (1994)	
b16-2	glucan endo-1,6-β-glucosidase	*T. harzianum*	Lora *et al.* (1995)	X79196
ura5	orotidine-5′-phosphate decarboxylase	*T. reesei*	Berges *et al.* (1990)	X55879
pkt1	serine/threonine protein kinase	*T. reesei*	Morawetz *et al.* (1994)	U05811
pgk-49	phosphoglycerate kinase	*T. viride*	Goldman *et al.* (1990)	X54284
chi42, pc1ch1	endochitinase	*T. harzianum*	Draborg *et al.* (1996)	U49455
5s rRNA	5S ribosomal RNA	*T. harzianum*	Ospina-Giraldo *et al.* (unpublished)	U58631
crea	DNA-binding protein	*T. reesei*	Takashima *et al.* (unpublished)	D63514

Table 9.1 *(Cont)*

Gene[a]	Product	Organism	Reference	Accession[b]
5.8s rRNA	5.8S ribosomal RNA	*T. longibrachiatum*	Ruiz-Sala *et al.* (1993)	L07957
bgn3.1	endo-1,3(4)-β-glucanase	*T. harzianum*	de la Cruz *et al.* (1995)	X84085
ind-a1	INDA1	*T. harzianum*	Vasseur *et al.* (1995)	Z22594
ind-c11	INDC11	*T. harzianum*	Vasseur *et al.* (1995)	Z22221
25s rRNA	25S ribosomal RNA	*T. reesei*	Vanhanen and Penttilä (unpublished)	X77580
5.8s rRNA	5.8S ribosomal RNA	*T. reesei*	Vanhanen and Penttilä (unpublished)	X77579
18s rRNA	18S ribosomal RNA	*T. reesei*	Vanhanen and Penttilä (unpublished)	X77581
actin	actin	*T. reesei*	Matheucci *et al.* (1995)	Z75421
cbh2	cellobiohydrase II	*T. reesei*	Stangl *et al.* (1993)	X70232 and S54964
endo51	endoglucanase I	*T. reesei*	Penttilä *et al.* (1986)	M15665
xyl1	arabinofuranosidase/β-xylosidase	*T. koningii*	Huang *et al.* (unpublished)	U38661
cre154	Cre1	*T. reesei*	Strauss *et al.* (1995)	U27356
18s rRNA	18S ribosomal RNA	*T. longibrachiatum*	Kuhls (unpublished)	Z31019
cons-b4	serine + alanine-rich protein	*T. harzianum*	Goldman *et al.* (1994)	Z22229

Table 9.1 *(Cont)*

Gene[a]	Product	Organism	Reference	Accession[b]
xln2	endoxylanase II	*T. reesei*	Saarelainen *et al.* (1993)	S67387
tham-ch	endochitinase	*T. hamatum*	Fekete *et al.* (unpublished)	Z71415
imid	imidazoleglycerol-phosphate	*T. harzianum*	Goldman *et al.* (1992)	Z11528 and S47086
qid3	putative catabolite-repressed protein	*T. harzianum*	Lora *et al.* (1994)	X71913
glucu1	α-glucuronidase	*T. reesei*	Margolles-Clark *et al.* (1996a)	Z68706
cell1	1,4-β-D-glucan cellobiohydrolase	*T. viride*	Cheng *et al.* (1990)	X53931
xyn1	endo-β-1,4-xylanase I	*T. reesei*	Torronen *et al.* (1992)	S51973
xyn2	endo-β-1,-4-xylanase I	*T. reesei*	Torronen *et al.* (1992)	S51975
trpl32	ribosomal protein L32	*T. harzianum*	Lora *et al.* (1993)	X71914
th1433	14.3.3.protein	*T. harzianum*	Harman and Hayes (unpublished)	U24158

[a] Most of the gene names were derived from the original articles. Some of them were assigned by us.
[b] Accession number for the GenBank/EMBL DNA Sequence data library.

Trichoderma spp. active in biocontrol (Herrera-Estrella *et al.,* 1993). From data based on gene location and DNA homology (as deduced from hybridization signals), the same authors have shown that *T. harzianum* and *T. viride* are closely related and could have evolved in the same phylogenetic branch, whereas *T. reesei* would most probably have derived from an independent branch. In another study, Mäntylä *et al.* (1992) determined molecular karyotypes of strains of *T. reesei* that had undergone mutagenesis and screening to produce strains that are hyperproducers of cellulase. These authors showed that rather extensive alterations in genome organization occurred in these strains.

9.3 Gene cloning

A large number of *Trichoderma* genes have been cloned (Table 9.1). These genes have been cloned using differential hybridization (Goldman *et al.,* 1994; Vasseur *et al.,* 1995), synthetic probes based on protein sequence data (Geremia *et al.,* 1994), heterologous gene probes (Heindenreich and Kubicek, 1994), a combination of synthetic oligonucleotides and PCR-based amplification (Hayes *et al.,* 1994) or complementation utilizing adequate expression vectors in *Saccharomyces cerevisiae* (Goldman *et al.,* 1992).

9.4 Translation control sequences

Kozak (1978) proposed a model for the initiation of translation in eukaryotes in which the ribosomal subunits can scan the messenger RNA from the 5' end and initiate translation at the first AUG triplet encountered. The context of the triplet is important, and indeed there is a high degree of conservation of the sequence around the initiator codon, GCC${}^{A}/_{G}$CC*AUG*G being the consensus in mammalian mRNAs (Kozak, 1987), ${}^{A}/_{Y}$A${}^{A}/_{U}$A*AUG*UCU in *Saccharomyces cerevisiae* (Cigan and

Table 9.2 Frequency of bases around the translation initiation codon[a]

	−10	−9	−8	−7	−6	−5	−4	−3	−2	−1	+4[b]	+5	+6
G	8	7	9	6	13	7	2	5	4	5	7	11	8
A	11	13	8	8	11	7	6	30	18	20	13	6	11
T	10	10	12	5	13	15	8	1	10	1	11	15	15
C	14	13	14	24	6	14	27	7	11	17	12	11	9
Consensus sequence[c]						${}^{T}/_{C}$	C	A	A	${}^{A}/_{C}$	${}^{A}/_{Y}$	${}^{T}/_{G}$	${}^{T}/_{A}$

[a] Data were compiled from the sequences listed in Table 9.1.
[b] The start codon AUG represents +1 to +3.
[c] The consensus sequence was assigned according to the following criteria: If the frequency of a single nucleotide is greater than or equal to 50% and greater than twice that of the second most abundant nucleotide, it is assigned as the consensus nucleotide and given in upper case. If the second criterion is satisfied but not the first, then the nucleotide is shown in lower case. If the sum of the frequencies is greater than 75% (but neither satisfies the above criteria), they are jointly assigned the status of the consensus.

Donahue, 1987), and $CA^{C}/_{A}{}^{A}/_{C}$*AUG*C in filamentous fungi (Ballance, 1991). Table 9.2 shows the frequency of bases around the translation initiation codon for genes from *Trichoderma* spp. Based on these data, the consensus sequence for mRNA in *Trichoderma* spp. is $^{T}/_{C}CAA^{A}/_{C}AUG^{A}/_{Y}{}^{T}/_{G}{}^{T}/_{A}$. The consensus sequence around the initiator codon in Table 9.2 shows that the *AUG* environment in *Trichoderma* spp. is also highly conserved. The most important position would appear to be -3 (the A of *AUG* being +1 and the preceding base being -1), at which a purine is nearly always present (97%) (Kozak, 1987) and usually as an A.

9.5 Codon usage

The knowledge of the pattern of codon usage in a genome has a number of practical applications in the investigation of the molecular genetics of that species, e.g. in interpreting sequence data and in designing oligonucleotide probes. Table 9.3 shows the codon frequencies in *Trichoderma* spp. Codon usage in *Trichoderma* spp. was evaluated in terms of two statistics. Firstly, codon usage was added up every time the codon was used. A more sophisticated measure is the Relative Synonymous Codon Usage (RSCU) measure of Sharp and Devine (1989). This is an expression of the number of times a particular codon is used relative to how often it is expected to be used if codon usage bias does not exist. RSCU values that are close to 1.00 indicate that the particular codon is being used at about the unbiased frequency. As the RSCU value moves further away from 1.00, either there is a bias for more frequent use of the codon (RSCU values greater than 1.00) or there is a bias against the use of this codon (RSCU values less than 1.00). Using RSCU values has the advantage of normalising codon usage for each codon. If a particular amino acid is used frequently in a dataset, the number of times that the encoding triplets are used will seem quite high (the converse is true for amino acids with low frequency of use). RSCU values are independent of amino acid usage and so looking at these values can give a better estimate of codon preference. In this dataset, for instance, the UUC codon for Phe and the CUC codon for Leu are both used approximately the same number of times (431 in the case of Phe and 436 in the case of Leu). However, the UUC codon is used 1.37 times more often than expected, whereas the CUC codon is used 2.17 times more frequently than in a situation where no bias exists. The converse is true for the UGU codon for Cys and the GGG codon for Gly. While the latter is used more frequently, its RSCU value is further from 1.00, so although UGU is not used very frequently, the pressure against using GGG is greater. The frequency of use of GGG is related to the frequency with which Gly appears in the proteins in this dataset. In general, the codons that end with a strong-bonding nucleotide (G or C) appear to be favored. The average G + C composition of the dataset is approximately 58%, while the average G + C base composition at the third position of codons for which there is a synonymous alternative (all codons except those encoding Met, Trp and the three termination codons) is 70%. It is obvious that mutational pressure towards an elevated G + C-content genome has a considerable effect on codon usage. The exceptions to this rule appear to be when the middle nucleotide of the triplet is strongly bonding. In these cases, there is discrimination against a G in the third position. This situation does not seem to exist for these C-ending codons and in all cases the C-ending codons are used more frequently than expected.

Table 9.3 Codon frequencies in *Trichoderma* spp. genes[a]

AA	Codon	N	RSCU	AA	Codon	N	RSCU	AA	Codon	N	RSCU	AA	Codon	N	RSCU
Phe	UUU	200	0.63	Ser	UCU	327	1.28	Tyr	UAU	163	0.51	Cys	UGU	62	0.42
	UUC	431	1.37		UCC	398	1.56		UAC	472	1.49		UGC	232	1.58
					UCA	109	0.43								
Leu	UUA	6	0.03		UCG	246	0.96	STOP	UAA	21	0.00	Trp	UGG	258	1.00
	UUG	127	0.63		AGU	71	0.28	STOP	UAG	14	0.00				
	CUU	193	0.96		AGC	381	1.49	STOP	UGA	6	0.00	Arg	CGU	146	1.27
	CUC	436	2.17										CGC	221	1.92
	CUA	35	0.17	Pro	CCU	267	1.08	His	CAU	75	0.40		CGA	112	0.97
	CUG	408	2.03		CCC	425	1.72		CAC	298	1.60		CGG	67	0.58
					CCA	107	0.43						AGA	53	0.46
Ile	AUU	252	0.96		CCG	191	0.77	Gln	CAA	140	0.39		AGG	91	0.79
	AUC	508	1.94						GAG	586	1.61				
	AUA	27	0.10	Thr	ACU	271	0.90					Gly	GGU	374	0.93
					ACC	553	1.83	Asn	AAU	169	0.36		GGC	896	2.23
Met	AUG	354	1.00		ACA	127	0.42		AAC	764	1.64		GGA	241	0.60
					ACG	255	0.85						GGG	95	0.24
Val	GUU	249	0.97					Lys	AAA	54	0.16				
	GUC	563	2.18	Ala	GCU	451	1.13		AAG	639	1.84	Glu	GAA	120	0.37
	GUA	34	0.13		GCC	758	1.90						GAG	529	1.63
	GUG	185	0.72		GCA	166	0.42	Asp	GAU	301	0.68				
					GCG	219	0.55		GAC	580	1.32				

[a] This table was compiled from a total of 17 109 codons (41 genes).

Sharp and Devine (1989) identified a small number of codons that appear to be "universally" preferred. These include the WWC codons (W = U or A): UUC (Phe), UAC (Tyr), AUC (Ile) and AAC (Asn). It appears that in *Trichoderma,* these codons are also used at a greater frequency than is expected and thus constitute preferred codons. The "universally optimum" UUC codon for Phe is used more than twice as often as the UUU codon. For Leu, the CUC and CUG codons are both used preferentially with both "A"-ending codons being rarely used. Of the three Ile codons, the AUA codon is only used one-tenth as often as expected, the AUU codon is used at about the expected frequency, and the AUC codon is preferred. Again the "A"-ending codon is rarely used to encode valine. The GUC codon is preferred and the GUA codon is used less than expected. This is probably due to the necessity for an optimum hydrogen-bonding interaction between the codon and its cognate amino-acyl tRNA. The GUG (strong-weak-strong) codon may involve a set of bonds that are too strong.

The codons that possess a "C" in the middle position and third position are used more often than expected. The codons that have a "C" in the middle position and a "G" in the third position do not appear to be favored (although their usage is only slightly less than the expected). The "A"-ending codons are used less than half as often as would be expected and the "U"-ending codons are used about as often as would be expected. When the strong-bonding "G" residue is found in the middle of a codon, there is reduced usage of the "G"-ending codons and a strong preference for the "C"-ending codons. These data suggest that the identity of the middle nucleotide of a triplet has a dramatic effect on the usage of the "G"-ending codons. When the middle nucleotide is weak-bonding (used two hydrogen bonds during duplex formation), there is a strong preference for the usage of "G"- and "C"-ending codons. When the middle nucleotide is strong-bonding (either an "A" or a "U"), then only the "C"-ending codons are preferentially used. The explanation for this probably lies either in steric hindrance or selection for more rapid translation of the codon. In cases where the codon-anticodon interaction is too strong, translation may be slowed down.

Of course the information in Table 9.3 does not take into consideration the variation within the dataset. The table merely presents a composite picture of the codon usage pattern for the dataset as a whole. It is necessary to use correspondence analysis to identify the major sources of variation in codon usage in the dataset. Correspondence analysis of a molecular dataset (usually carried out on the RSCU values) seeks to identify the major source of variation within the dataset. Each gene is assigned a position on a 59-dimensional axis, so constructed because there are 59 codons for which there is an asynonymous alternative (excluding the three STOP codons and Trp, which is encoded by UGG). If there is no codon usage bias, the "cloud" formed by the points representing the genes will appear spherical. If there is a codon usage trend (from high GC to low GC; or from high Effective Number of Codons (ENC) values to low ENC values; or from a high abundance of A in the first position to a high abundance of T in the first position;…or whatever), then the "cloud" will no longer look spherical but will assume a sausage-like shape. The axis that goes through the middle of this "sausage" is the axis that "corresponds" to the major source of variation in the dataset (the most important factor of dispersion). At one end of the axis are the genes with high codon bias and the genes with low codon bias are at the other end. The computer programs do scaling according to gene length and other considerations, but these are not of

Table 9.4 Correspondence analysis to identify the major sources of variation in *Trichoderma* spp. codon usage

Gene name	AX1	Laa	GC	GC3s	ENC
xyn1	−47	222	0.62	0.88	33.7
xln2	−47	223	0.62	0.89	30.7
ura5	−43	236	0.62	0.86	32.6
eg11	−41	463	0.65	0.90	33.1
hfb1	−41	97	0.65	0.78	29.6
imid	−38	208	0.66	0.85	34.7
indc11	−35	339	0.62	0.83	34.4
pk11	−34	538	0.61	0.82	31.9
eg15	−33	242	0.65	0.77	41.2
endo51	−29	459	0.63	0.86	37.2
tef1	−18	460	0.59	0.79	26.8
th1433	−15	262	0.59	0.77	34.9
tub2	−10	446	0.58	0.75	33.1
pgk49	−7	423	0.59	0.79	35.5
cre154	−7	402	0.62	0.73	41.8
pkt1	−7	662	0.59	0.76	43.8
cbh1	−6	513	0.59	0.73	40.7
crea	−6	402	0.62	0.73	41.9
pkc1	0	1139	0.60	0.73	45.8
b16-2	1	60	0.57	0.64	46.6
inda1	3	573	0.56	0.67	39.8
glucu1	5	847	0.57	0.71	48.0
celll	7	513	0.59	0.70	44.3
chit33	7	321	0.56	0.67	44.5
actin	9	366	0.56	0.69	33.6
cre1	9	409	0.60	0.68	46.1
pyr	9	379	0.58	0.67	50.5
tub1	11	446	0.55	0.64	40.6
xyn2	12	229	0.55	0.63	46.6
bgl1	15	744	0.58	0.67	49.0
xyl1	16	500	0.58	0.62	50.2
chi42, pc1ch1	23	424	0.54	0.67	41.8
endchi1	29	428	0.53	0.62	44.9
ech2	31	424	0.53	0.65	43.9
cbhII	32	471	0.55	0.56	55.1
consb-4	32	170	0.66	0.58	38.4
trp 132	34	137	0.58	0.66	46.5
eg3	40	418	0.53	0.56	55.9
prb1	42	409	0.52	0.49	44.8
axe1	43	302	0.58	0.58	57.0
bgn3	52	762	0.51	0.49	54.0

AX1: Position on the axis of greatest dispersion.
Laa: The number of amino acids in the gene.
GC: The G + C base composition summed over all positions.
GC3s: The G + C base composition at the third position of codons that have a synonymous alternative.
ENC: Effective number of codons.

Table 9.5 Results of the chi-squared test for significant differences between the RSCU values for the highly biased dataset and the lowly biased dataset (asterisk indicates a codon that is used significantly more frequently)

AA	Codon	N	RSCU	N	RSCU	AA	Codon	N	RSCU	N	RSCU
Phe	UUU	15	0.70	27	0.75	Ser	UCU	10	0.48	43	1.34
	UUC	28	1.30	45	1.25		UCC*	43	2.08	38	1.19
							UCA	1	0.05	34	1.06
Leu	UUA	1	0.09	3	0.15		UCG*	30	1.45	21	0.66
	UUG	1	0.09	26	1.27		AGU	2	0.10	13	0.41
	CUU	3	0.26	30	1.46		AGC	38	1.84	43	1.34
	CUC*	36	3.13	30	1.46						
	CUA	2	0.17	5	0.24	Pro	CCU	7	0.43	42	1.53
	CUG*	26	2.26	29	1.41		CCC*	33	2.03	29	1.05
							CCA	2	0.12	22	0.80
Ile	AUU	14	0.74	44	1.21		CCG*	23	1.42	17	0.62
	AUC*	42	2.21	58	1.60						
	AUA	1	0.05	7	0.19	Thr	ACU	11	0.44	59	1.27
							ACC*	56	2.22	71	1.53
Met	AUG	17	1.00	23	1.00		ACA	3	0.12	30	0.65
							ACG*	31	1.23	26	0.56
Val	GUU	6	0.31	54	1.69						
	GUC*	54	2.81	43	1.34	Ala	GCU	18	0.71	85	1.73
	GUA	0	0.00	11	0.34		GCC*	63	2.50	54	1.10
	GUG	17	0.88	20	0.62		GCA	2	0.08	34	0.69
							GCG	18	0.71	23	0.47
Tyr	UAU	2	0.06	32	1.05						
	UAC*	64	1.94	29	0.95	Cys	UGU	1	0.06	19	0.97
							UGC*	31	1.94	20	1.03
stop	UGA	2	0.00	1	0.00						
stop	UAA	2	0.00	3	0.00	Trp	UGG	19	1.00	38	1.00
stop	UAG	1	0.00	1	0.00						
						Arg	CGU	3	0.48	24	1.80
His	CAU	2	0.29	19	0.93		CGC*	26	3.63	13	0.98
	CAC*	12	1.71	22	1.07		CGA	0	0.00	14	1.05
							CGG	8	1.12	10	0.75
Gln	CAA	4	0.17	39	0.83						
	CAG*	44	1.83	55	1.17	Arg	AGA	0	0.00	8	0.60
							AGG	6	0.84	11	0.83
Asn	AAU	6	0.14	42	0.62						
	AAC*	79	1.86	94	1.38	Gly	GGU	9	0.25	62	1.12
							GGC*	115	3.22	96	1.74
Lys	AAA	3	0.13	10	0.36		GGA	7	0.20	52	0.94
	AAG	42	1.87	45	1.64		GGG	12	0.34	11	0.20
Asp	GAU	11	0.42	42	1.01	Glu	GAA	3	0.15	18	0.88
	GAC*	42	1.58	41	0.99		GAG*	36	1.85	23	1.12

Chi-squared values follow for optimal codons:

Codon UCC = 8.758	Codon CGC = 25.250	Codon ACG = 11.490
Codon UAC = 39.507	Codon CUG = 4.302	Codon GUC = 25.746
Codon UGC = 18.058	Codon CCG = 9.204	Codon GCC = 33.858
Codon UCG = 9.782	Codon CAG = 16.545	Codon GAC = 12.116
Codon CUC = 15.126	Codon AUC = 6.550	Codon GGC = 48.731
Codon CCC = 10.638	Codon ACC = 7.917	Codon GCA = 13.537
Codon CAC = 4.543	Codon AAC = 17.461	

major importance. The function of the analysis for any dataset is to identify why deviations from the spherical cloud occur, such as base and so on.

In every organism that has been examined to date, it has been shown that not all codons are used with equal frequency in all of the genes of the organism. Correspondence analysis finds the major source of variation in a dataset; at one end of this axis are the genes in which the greatest codon bias occurs. This amount of selectivity might be trivial, so a chi-squared test is used to see if there is a significant difference between the usage of codons in genes where less bias exists. Hereafter, "highly biased" will indicate that codon usage in that gene is more strongly biased than average. The reason for its position must be investigated to see if it is related to another statistic such as GC3s or ENC or position on the chromosome, etc. The significance of this phenomenon is that if a species exhibited a large long-term effective population size and is not subject to appreciable random genetic drift, then it will have had enough time to streamline its codon usage into an efficient means of rapidly translating mRNA. It will evolve a more biased codon usage pattern, which gives it a better chance of incorporating the correct tRNA (the population of which will also have reduced diversity) into the growing chain more quickly. The genes that benefit most from this kind of behavior are the highly expressed genes which exert a stronger selective pressure on the organism. In most prokaryotes and yeast we see the greatest bias in the highly expressed genes. In mammals, for instance, which have small, long-term effective population sizes and are subject to the vagaries of random genetic drift and frequent extinction, the codon usage is merely a reflection of the GC content of the region of DNA in which the gene resides. If we know which pattern a particular organism is likely to have, we can predict what the codon usage pattern for a particular (unknown) gene might be.

Thus, correspondence analysis was performed to identify the major sources of variation in *Trichoderma* spp. codon usage (Table 9.4). The genes are arranged in order of their appearance on the axis of greatest dispersion. The genes at the top of the table are the more biased genes and the genes towards the bottom of the table are less biased. In order to examine whether there was a difference in the usage of codons in the genes from either end of the axis of greatest dispersion, a total of five genes were selected from either end (1246 codons from one end and 2033 from the other). The cumulative RSCU values for each set were compared and a chi-squared test for heterogeneity within amino acid groups was carried out to the level of $P < 0.01$. A total of 20 codons were used significantly more frequently in the highly biased set than in the lowly biased set. This is an indication of the considerable amount of variation in codon usage within the dataset. The results of this analysis are shown in Table 9.5, with an asterisk denoting the codons that are used significantly more often in the highly biased dataset.

9.6 Conclusions

For *Trichoderma* spp., the estimated genome sizes range from 31 to 39 Mb and chromosome numbers range from 3 to 7. This large variation can be explained by assuming the hypothesis that variation in numbers and sizes of chromosomes is tolerated in imperfect fungi because meiosis does not occur and so chromosome pairing is unnecessary (Harman *et al.,* 1993; Kistler and Miao, 1992). Well over 50 genes from *Trichoderma* spp. have now been isolated and characterized. Their DNA

sequences have revealed the presence of a number of common sequence elements that might be important in the expression of these genes. We have shown an initial summary of their gene structure. Our analyses put more emphasis on translational rather than transcriptional signals. Further research on transcriptional signals will need more functional analysis *in vivo* and *in vitro*. Other points of interest for analysis are RNA splicing signals (intron splice junctions and internal consensus sequences), presence of signal peptides and DNA regions important for gene regulation. Some of these topics are currently under investigation in our laboratory.

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Genetic transformation of *Trichoderma* and *Gliocladium*

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10.1 Introduction

A major prerequisite for molecular genetic research in filamentous fungi is the availability of a gene transfer system. This consists of two main components: a vector containing a selectable marker and a transformation procedure to introduce the corresponding vector into the fungus. There has been substantial progress in developing transformation techniques for filamentous fungi during the last 25 years. Besides the unicellular yeast *Saccharomyces cerevisiae,* the filamentous fungi *Neurospora crassa* and *Aspergillus nidulans* have been studied intensively. Several excellent reviews on transformation systems and genetic engineering of filamentous fungi have been published (Fincham, 1989; Goosen *et al.,* 1991; Timberlake and Marshall, 1989; Turner, 1991; Van den Hondel and Punt, 1991). The molecular genetic systems of the two model organisms of filamentous fungi (*N. crassa* and *A. nidulans*) served as the basis for developing suitable transformation systems for less investigated but economically and industrially important fungal species (Timberlake and Marshall, 1989).

The first fungal transformation was reported in 1973 by Mishra and Tatum. An inositol-requiring mutant of *N. crassa* was transformed with total DNA of the wild type by the addition of calcium chloride and the wild-type DNA to a growing culture. Conidia that formed after this treatment were selected for prototrophy. A few years later, Hinnen *et al.* (1978) became the first to perform transformation of a *leu2*-mutant of *S. cerevisiae* using the protoplast technique. Again, treatment with wild-type DNA in the presence of calcium chloride led to the formation of prototrophic strains. The protoplast technique, which already was proven to be a sufficient and useful tool for yeast transformation, was immediately applied to the filamentous fungi *N. crassa* and *A. nidulans* (Case *et al.,* 1979; Tilburne *et al.,* 1983).

Throughout the years, transformation techniques were developed for other fungal species including some *Trichoderma* and *Gliocladium* species (Table 10.1).

Table 10.1 Overview of different markers used for *Trichoderma* and *Gliocladium* spp.

Species	Markers	Reference
Trichoderma reesei	*amdS*, *argB*	Penttilä *et al.* (1987b)
Trichoderma reesei	*ura3*, *ura5*	Bergès and Barreau (1991)
Trichoderma reesei	*pyrG*	Gruber *et al.* (1990a)
Trichoderma reesei	*pyr4*	Gruber *et al.* (1990b) Smith *et al.* (1991)
Trichoderma reesei	*hph*	Mach *et al.* (1994)
Trichoderma viride	*pyr4*	Cheng *et al.* (1990)
Trichoderma viride	*hph*, *tub2*	Herrera-Estrella *et al.* (1990); Goldman *et al.* (1992)
Trichoderma harzianum	*hph*, *bml*	Herrera-Estrella *et al.* (1990), Uhloa *et al.* (1992), Sivan *et al.* (1992), Goldman *et al.* (1993)
Trichoderma harzianum	*amdS*	Pe'er *et al.* (1991)
Trichoderma hamatum	*hph*, *bml*	Uhloa *et al.* (1992)
Gliocladium roseum	*hph*	Thomas and Kenerly (1989)
Gliocladium virens	*hph*	Thomas and Kenerly (1989)

10.2 Transformation procedures

Most frequently, protoplasts are used for introduction of exogenous DNA. Protoplasts are obtained by incubating mycelia or spores with different cell wall degrading enzymes under osmotically stabilized conditions. An excellent overview for different procedures and enzymes used for protoplasting is described by Peberdy (1989). Besides the generally used calcium chloride/PEG (polyethylene glycol) method, an electroporation method for protoplasts was described by Thomas and Kenerley (1989) and by Goldman *et al.* (1990). In addition to these standard methods, a few reports describe the use of intact cells for transformation. Recently, particle bombardment has successfully been used for transformation of *Trichoderma* and *Gliocladium* spp. (Lorito *et al.,* 1993).

10.2.1 Polyethylene glycol-mediated transformation

Polyethylene glycol (PEG)-mediated transformation technique is the most frequently used for filamentous fungi. The critical step of this method is the requirement of protoplasts that are produced by removing the cell walls of germ tubes or hyphae with cell wall degrading enzymes. Most commonly an enzyme mixture, commercially available as Novozyme 234 (Novo Nordisc) which is secreted by the filamentous fungus *Trichoderma harzianum,* is used for protoplasting. Sometimes lytic enzymes from *Trichoderma harzianum* are combined with others such as *ß*-glucuronidase and chitosanase. However, during the whole preparation procedure of protoplasts, the presence of osmotic stabilizers is required. In the first protocol for protoplasting described for *Trichoderma reesei* by Penttilä *et al.* (1987b), osmotic stability was achieved by the use of sorbitol at a concentration of 1.2 M. An alternative method (osmotic stabilisation with 1.2 M $MgSO_4$) was described in the same publication.

In general, sorbitol and $MgSO_4$ are most commonly used but, for some fungi, satisfying results are also achieved with mannitol or sodium chloride (Fincham, 1989). In the case of *Trichoderma reesei,* 1.0 to 1.2 M sorbitol has proven to function as a good osmotic stabilizer for protoplasts as shown by a regeneration rate of over 90%.

In a standard protocol for PEG-mediated transformation, protoplasts at a concentration of 5×10^7–5×10^8 are treated with a mixture of calcium chloride, PEG and transforming DNA as essentially described for *A. nidulans* by Ballance *et al.* (1983) and Tilburne *et al.* (1983). The exogenous DNA molecules apparently are internalized while a PEG-induced protoplast fusion takes place (no transformation occurs when PEG is omitted) (Timberlake and Marshall, 1989). After transformation, the protoplasts are plated on appropriate regeneration media with an overlay that contains a toxicant or other selective agent. Bottom and overlay media are osmotically stabilized and are chosen to enable selection for the phenotype transmitted by the transforming DNA.

Penttilä *et al.* (1987b) applied a similiar procedure to that described above for *T. reesei*. After PEG treatment the regeneration frequency of protoplasts dropped from 90% to 12–35%. The authors reported a frequency of 150–400 transformants per μg of DNA for the complementation of an arginine-auxotrophic strain with the *argB* gene (Berse *et al.,* 1983) from *A. nidulans* and a frequency of up to 600 transformants per μg of DNA with selection based on the acetamidase gene of *A. nidulans* (Hynes *et al.,* 1983).

The major disadvantages of the PEG-mediated transformation technique are the variability of the efficiency of protoplast formation and regeneration for different strains. Because storage of protoplasts will drastically reduce the transformation frequency (Penttilä *et al.,* 1987b), protoplasts have to be prepared freshly for each transformation experiment. In addition to these problems, protoplasts often bear more than one nucleus leading to a long and time-consuming purification of transformants to obtain homokaryons.

10.2.2 Lithium acetate method

The lithium acetate method was first developed as an alternative transformation system for *S. cerevisiae*. Ito *et al.* (1983) tried a variety of cations and found that 0.1 M lithium acetate gave the best results. In this method, protoplasting is avoided by using high alkaline metal ion concentrations to induce DNA permeability. This procedure has also been used successfully for *N. crassa* (Dhawale *et al.,* 1984) and *Coprinus cinereus* (Binninger *et al.,* 1987). In both cases germinating spores were treated with 0.1 M lithium acetate and transforming DNA. The mechanism by which alkali metals assist uptake of the transforming DNA is not clear. This technique is not frequently used for filamentous fungi and has not been described for *Trichoderma* or *Gliocladium* species.

10.2.3 Electroporation

Use of electroporation has been reported for both pro- and eukaryotic cells (Förster and Neumann, 1989; Miller *et al.,* 1988). Some reports describing electroporation

to transform filamentous fungi are available. Protoplasts of *A. awamori* and *A. niger* were transformed by electroporation and the frequency obtained was similiar to that obtained with PEG technique (Ward *et al.,* 1989). Goldman *et al*. (1990) developed conditions for an efficient method of genetic transformation in *T. harzianum* using high-voltage electroporation. In this protocol, "competent cells" were prepared by partial digestion of the fungal cell wall to provide osmotically sensitive cells. In order to induce cells to take up transforming DNA, an electric pulse and PEG were used in the electroporation medium. Frequencies of up to 400 transformants per μg of DNA could be obtained. These frequencies seem to be comparable to the transformation efficiencies previously reported for *Trichoderma* spp. by Herrera-Estrella *et al.* (1990) and for *Trichoderma reesei* by Penttilä *et al.* (1987b), using PEG- and $CaCl_2$ mediated DNA transformation. The two advantages of electroporation described by Goldman *et al.* (1990) over the traditional methods are (a) the simplicity of preparing osmotically sensitive cells (they do not have to be purified on a sorbitol gradient) and (b) a higher reproducibility of transformation using this method. An additional important difference observed was an increase of mitotical stability of transformants from *T. harzianum*.

10.2.4 Particle bombardment

Particle bombardment as described by Klein *et al.* (1987) employs high-velocity microprojectiles to deliver nucleic acids into intact living cells and tissues. This method has also been called microprojectile bombardment, gene gun method, particle-acceleration method or biolistic transformation. The procedure involves coating the DNA on microbeads composed of tungsten or gold. The sizes of beads used range from 0.5 to 5 μm, depending on the dimensions and penetrability of the target cells. A few μL of a slurry of these microprojectiles are placed on the surface of a macroprojectile that is accelerated by gun powder or helium discharge. A stopping plate at the end of a barrel stops the macroprojectile but a small hole or a net allows the microprojectiles to proceed toward the target cells. In currently available equipment, the microprojectiles are placed on a membrane from which they are accelerated to target cells by a helium charge. Usually the bombardment takes place in a vacuum chamber in order to lessen the impediment of air on the velocity of microprojectiles. The device and procedure were originally applied to plants (Klein *et al.,* 1988a–c; Wang *et al.,* 1988) and afterwards also to mammals (Williams *et al.,* 1991), bacteria (Shark *et al.,* 1991) and fungi like *S. cerevisiae, S. pombe*, and *N. crassa* (Armaleo *et al.,* 1990). Armaleo *et al.* (1990) indicate that this technique of delivering nucleic acids into fungal cells is becoming widespread. Detailed description of the biolistic transformation of *T. harzianum* and *G. virens* is reported by Lorito *et al.* (1993). This article compared protoplast-mediated and biolistic transformation relative to stability of integration and transformation frequencies. Biolistic procedures led, for both *Trichoderma* and *Gliocladium,* to improvement in transformation frequency. Yields of about 600–800 transformants per μg of plasmid DNA for both fungi were obtained, which showed a 30% increase in transformation frequency compared to the standard protoplast method. Transformation with genomic DNA was successful using the biolistic method but was significantly less efficient than transformation with plasmid DNA. Further, transformants were more stable following biolistic as opposed to protoplast transformation. This may

be due to the fact that conidia contain fewer or single nuclei than protoplasts and therefore the percentage of heterokaryotic transformants should decrease with the biolistic transformation procedure. A comparison of transformed nuclei relative to biolistic versus the standard protoplast method exhibited 4–10 times more transformed nuclei in the case of the biolistic method (Lorito *et al.,* 1993).

In addition to the already mentioned advantages, the simplicity of application of this method allows for a high number of transforming experiments in a short time. Both steps, plating of conidia and coating of microprojectiles with DNA, are simple and therefore easy to standardize. For biolistic transformation there seems to be no limit to particular species because this technique has been employed successfully to organisms already transformable by other means but also to less and no transformable systems. Last but not least, the biolistic technique is so far the only one described for transformation of mitochondria and chloroplasts (Daniell *et al.,* 1990; Fox *et al.,* 1988; Johnston *et al.,* 1988).

10.3 Transforming DNA

10.3.1 Selection markers

Expression of a dominant selectable phenotype permits the selection of transformed cells within a background of non-transformed cells (Timberlake and Marshall, 1989). For selection of transformed cells three different types of selectable markers are applied: (a) genes coding for suppressor tRNA, (b) auxotrophic markers and (c) dominant selection markers.

In addition, a suppressor tRNA gene (*su*-8), which is presumed to be a mutated tRNA gene, was used as a selection marker for transformation of *Podospora anserina* (Brygoo and Debuchy, 1985). This marker may be applied for transformation of fungal strains that contain a suppressible chain termination mutation.

In early work, most transformations were carried out by converting auxotrophic mutants to prototrophic strains (Fincham, 1989). Obviously, a prerequisite for the successful use of this commonly applied selection method is the presence of appropriate mutants. Two-way selection systems are a useful tool to gain appropriate mutants, such as uridine-negative mutants selected via loss of orotidine-5'-monophosphate carboxylase activity (encoded by *ura3* or *pyr4* and required for uridine biosynthesis) which confers resistance to the inhibitory analogon 5-fluoro-orotic-acid (Alani *et al.,* 1987; Bergès and Barreau, 1991; Diez *et al.,* 1987; Gruber *et al.,* 1990b; Smith *et al.,* 1991). Besides the *pyr4* system, the *niaD* gene, which codes for nitrate reductase first characterized in *A. nidulans,* is an attractive selection marker for developing a gene transfer system for species that are not well characterized genetically. In this case, similar to the two-way *pyr4* system described above, loss of *niaD* function can be selected by resistance against chlorate (Unkles *et al.,* 1989). For *Trichoderma* spp., the reported auxotrophic transformation systems are the complementation of uridine-auxotrophic mutants of *T. reesei* via the genes *pyr4* or *ura3* and *ura5* (Bergès and Barreau, 1991; Gruber *et al.,* 1990b; Smith *et al.,* 1991) and the complementation of an arginine-auxotrophic mutant of *T. reesei* (Penttilä *et al.,* 1987b). This may be due to the fact that in the best investigated *Trichoderma* strain, *T. reesei,* no utilization of nitrate was observed and therefore selection for chlorate-resistant mutants was not possible.

As already mentioned, one of the obvious disadvantages of auxotrophic markers is the need to isolate appropriate recipient strains. To overcome this problem, a set of dominant selectable markers was developed that allows both transformation of wild-type and mutant strains (e.g. industrial strains). Dominant selectable markers utilized for different fungal species are given in Table 10.2.

Most of the markers mentioned in Table 10.2 are "broad host range", which can be applied to different fungal species. Most of them are based on drug resistance, either consisting of mutated fungal genes such as those encoding benomyl-resistant *ß*-tubulin, or bacterial antibiotic resistance genes under control of expression signals from filamentous fungi. An example of a specially designed vector for the transformation of *Trichoderma* containing a bacterial resistance gene *(hph)* is shown in Figure 10.1. In this case the hygromycin B-resistance gene *(hph)* was introduced in a fungal

Table 10.2 Overview of selectable markers used for transformation in filamentous fungi

Marker genes	Species of origin	Encoded function	Transformed species	Reference
amdS	*Aspergillus nidulans*	acetamidase	*Aspergillus niger*[a,b]	Kelly and Hynes (1985)
bar	*Streptomyces hygroscopicus*	phosphinothricin acetylase	*Neurospora crassa*	Avalos *et al.* (1989)
benA	*Aspergillus nidulans*	benomyl resistant *β*-tubulin	*Aspergillus nidulans*	May *et al.* (1985)
ble	*Escherichia coli*	phleomycin-binding protein	*Penicillium chrysogenum*[a]	Kolar *et al.* (1988)
ble	*Streptoalloteichus hindustanus*	phleomycin-binding protein	*Aspergillus nidulans* and *A. niger*[a]	Mattern *et al.* (1988)
bml	*Neurospora crassa*	benomyl resistant *β*-tubulin	*Neurospora crassa*[a,b]	Orbach *et al.* (1986)
G418[r]	*Escherichia coli*	geneticin/neomycin/ kanamycin phosphotransferase	*Ustilago maydis*[a]	Banks (1983)
hph	*Escherichia coli*	hygromycin B phosphotransferase	*Cephalosporium acremonium*[a,b]	Queener *et al.* (1985)
oliC	*Aspergillus nidulans*	mitochondrial ATP-synthase subunit 9	*Aspergillus nidulans*	Ward *et al.* (1986)
oliC	*Aspergillus niger*	mitochondrial ATP-synthase subunit 9	*Aspergillus niger*	Ward *et al.* (1988)
oliC	*Penicillium chrysogenum*	mitochondrial ATP-synthase subunit 9	*Penicillium chrysogenum*	Bull *et al.* (1988)
sul1	*Escherichia coli*	dihydropteroate synthase	*Penicillium chrysogenum*	Carramolino *et al.* (1989)
tub	*Colletotrichum graminicola*	benomyl-resistant *β*-tubulin	*Colletotrichum craminicola*	Panaccione *et al.* (1988)
tubA	*Septoria nodorum*	benomyl-resistant *β*-tubulin	*Septoria nodorum*[a]	Cooley and Caten (1989)

[a] indicates that the listed species is the first transformed with the corresponding marker.
[b] indicates marker already applied for transformation of *Trichoderma* spp. and/or *Gliocladium* spp., previously outlined in Table 10.1.

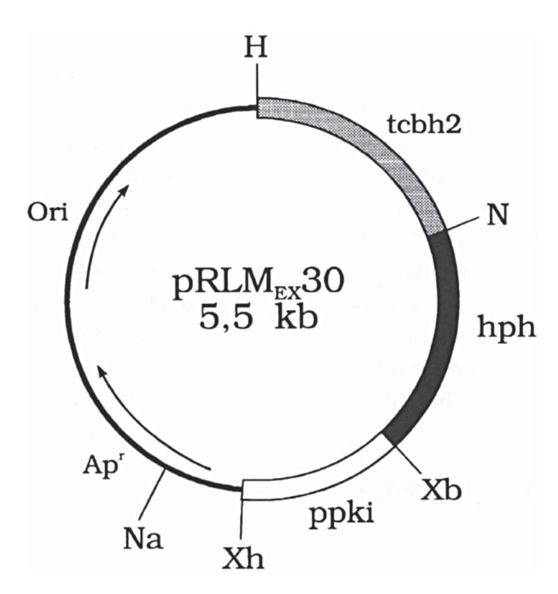


Figure 10.1 Vector designed for transformation of *Trichoderma* spp. bearing the dominant selection marker *hph* (hygromycin B phosphotransferase) under the control of *Trichoderma reesei* regulatory sequences: *ppki* (pyruvate kinase promoter) and *tcbh2* (cellobiohydrolase II terminator). The vector was developed from plasmid pUC19 and some single restriction sites are indicated. Xh *(XhoI),* Xb *(XbaI),* N *(NsiI),* H *(HindIII)* and Na *(NarI).*

expression vector bearing a promoter region of the highly expressed *T. reesei pki* gene and the terminator region of the *T. reesei cbh2* gene (Mach *et al.,* 1994).

A second drug resistance gene, a benomyl resistant *ß*-tubulin gene *bml* from *N. crassa,* was used successfully for transformation of *T. hamatum* and *T. harzianum* by Uhloa *et al.* (1992). The only exception in the list of dominant selection markers conferring resistance to toxicants is the acetamidase gene *(amdS)* of *A. nidulans* (Kelly and Hynes, 1985), which functions as a utilization marker. Transformants containing this gene are able to use acrylamide or acetamide as sole nitrogen and carbon sources. Generally, fungi cannot readily use these compounds and this transformation system has been applied to a wide variety of fungal species, including *T. reesei* (Penttilä *et al.,* 1987b) and *T. harzianum* (Pe'er *et al.,* 1991).

10.3.2 Cotransformation

In most cases, selection for transforming genes cannot directly be done. One possibility to overcome this problem is to look for assimilation along with a more readily selectable marker. If recipient cells are treated with two different kinds of DNA, the probability of taking up both is quite high (Fincham, 1989). This phenomenon of "cotransformation" was first investigated by Wernars *et al.* (1987). The authors could show that not all protoplasts take up DNA equally, but those most competent to do so simultaneously take up several molecules. The uptake of both transforming DNAs can be increased if the ratio of cotransforming to transforming DNA is kept high. Frequencies of reported cotransformation vary upon a broad range and therefore

seem to be dependent upon the organism and the cotransformation conditions. Penttilä *et al.* (1987b) reported the first cotransformation for *T. reesei* by using two selectable markers on two different plasmids in one procedure. Equal amounts of **DNA** were supplied and resulted in a cotransformation frequency of 68%. Kubicek-Pranz *et al.* (1991) first applied cotransformation to insert a selectable marker in parallel with a non-selectable gene and reported a cotransformation rate of about 50% using a ratio of 1:15 of cotransforming to transforming vectors. In their study they observed a very high copy number of the transforming plasmid which is in contrast to the results published by Harkki *et al.* (1991) and Uusitalo *et al.* (1991) who obtained *T. reesei* strains bearing only two copies of integrated vector DNA. The lower copy number of their transformants may be explainable from their transformation protocols; the authors used equal amounts of both plasmids.

10.4 Purification and characterization of transformants

10.4.1 Purification of mitotically stable transformants

Since most protoplasts or other structures in *Trichoderma* are multinucleate, in every protoplast-mediated transformation heterokaryotic transformants will occur. Selection has to be applied to purify individual nuclei from these heterokaryons. If the fungal species forms uninucleate conidia, e.g. *Aspergillus* spp., purification can very simply be performed by plating conidia and isolating single colonies. In *Trichoderma* spp., conidia are reported to be polynucleate and some conidia of *Gliocladium* spp. also contain multiple nuclei. For *T. harzianum,* data have been presented that polynucleate conidia appear as a consequence of proliferation as the conidia mature. Conidia newly formed and released from phialides are mononucleate (Stasz *et al.,* 1988). As commonly not all nuclei are transformed, genetic purification still has to be achieved by plating conidia and isolating single colonies. After three rounds the probability of stochastic loss of one or another nuclear component is high (Fincham, 1989). If selective and non-selective conidiation steps are interchanged, the loss of non-stable transformants, e.g. those bearing nuclei with non-integrated vectors, will occur and will lead to homokaryotic and mitotically stable strains.

10.4.2 Integration of DNA into the chromosome

Almost all commonly used plasmids for transformation of filamentous fungi have no origin of replication. In *Trichoderma* and *Gliocladium* spp., no vectors with autonomously replicating sequences are reported. Biochemical analyses of the chromosomal DNA of transformants indicate that, in general, three types of integration events can occur:

- type 1: integration of the vector by homologous recombination
- type 2: ectopic integration by non-homologous recombination
- type 3: gene replacements

In the case of type 1, part of the plasmid recombines at a region of homology within the genome (Figure 10.2A). For different organisms, a high variability of this type of

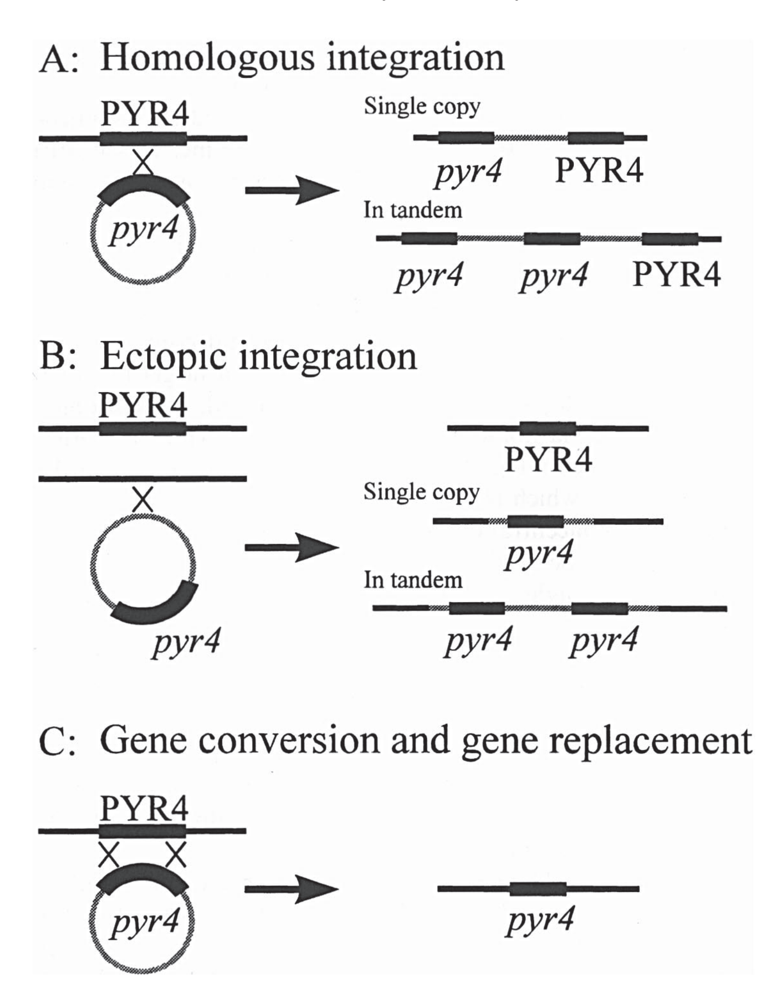


Figure 10.2 Integration types of transforming DNA into the genome.

integration event relative to others is described, e.g. *A. nidulans* is about 80% (Yelton *et al.,* 1984) and *N. crassa* is 1–5% (Case, 1986). Observations similiar to those with *N. crassa* were made for *T. reesei* where homologous integration frequency is reported at about 2% (Mach *et al.,* 1995; Seiboth *et al.,* 1992).

Type 2 is characterized by integration of the plasmid into the genome at a random position with no known homology (Figure 10.2B). Heterologous integration is presumably favoured by plasmids containing low amounts of DNA homologous to the target genome. It can be speculated that small stretches of target-plasmid DNA identity could provide nucleation sites to initiate a recombination reaction. Occurrence of these sites depends on their sizes and will lead to random integration (Timberlake, 1991). No systematic studies, at least for *Trichoderma* and *Gliocladium,* have been undertaken to determine if so-called ectopic integration of plasmids occurs by homologous recombination between short DNA sequences. Diallinas and Scazzocchio (1989) showed that a plasmid integrating into the *A. nidulans* genome at heterologous loci had clear site preferences and therefore was not

random. However, the sequence at the junctions between the integrated plasmids and the genome was not determined.

Type 3 takes place when the gene is aberrantly replaced by the introduced copy but no plasmid sequence can be detected within the genome. This is attributed to a homologous interaction between a part of the plasmid and the genomic DNA leading to gene conversion (Figure 10.2C).

Multicopy integration of plasmids caused by tandemly repeated copies is a widespread phenomenon in filamentous fungi. There are several possible explanations. First, this may be due to successive rounds of homologous integrations either into the homologous chromosomal locus or into the plasmid copy already integrated. Second, an ectopic integrated plasmid may function as a target for further integration leading to tandem ectopic plasmid integration. Third, extrachromosomal plasmids first concatemerize via homologous recombination with each other and these circular oligomers can then integrate into the chromosome (Fincham, 1989). Multicopy ectopic integration, which is the most prominent event in *Trichoderma* spp., is elevated by high DNA concentration and the use of circular plasmids.

Linear DNA also can be used for transformation and was first described for *S. cerevisiae* and effected a higher transformation efficiency relative to circular DNA (Suzuki *et al.,* 1983). In the case of filamentous fungi, no effect or even a lower transformation frequency was observed when transforming with linear DNA. Herrera-Estrella *et al.* (1990) reported a significantly lower transformation frequency in *Trichoderma* spp. relative to circular plasmid transformation. However, the use of linear molecules significantly increases homologous recombination events (Aramayo *et al.,* 1989; Boylan *et al.,* 1987). Several authors applied this technique for gene replacement or gene deletion in *T. reesei* (Fowler and Brown, 1992; Mach *et al.,* 1995; Seiboth *et al.,* 1992; Suominen *et al.,* 1993).

Genetic manipulation of filamentous fungi strictly is dependent on the incorporation of the added DNA, e.g. the transforming plasmid. Mitotically stable plasmids are often meiotically unstable. Tandem repeat sequences are sometimes lost after cell fertilization or outcrossing in *A. nidulans* (Tilburne *et al.,* 1983). Very high frequencies of elimination of duplicate sequences are reported for *N. crassa* by the RIP process (repeated induced point mutation) (Selker *et al.,* 1987; Selker and Garrent, 1988). Similiar observations have until present not been reported for *Trichoderma* and *Gliocladium,* which may be due to the fact that most strains used for laboratory purposes lack a sexual phase. Lorito *et al.* (1993) found methylation events within the integrated vector DNA used for biolistic transformation of *T. harzianum* and *G. virens*. The authors speculated that this could be an effect of specific modification of duplicate sequences similiar to the RIP effect described above. No clear evidence for this postulation was observed.

10.5 Applications in molecular biology and biotechnology

As already mentioned, transformation systems and integration of the transforming DNA are basic tools for research based on molecular biology and for strain improvement for biotechnological applications. This chapter will only outline two examples for applying transformation systems. Additional information upon further fruitful utilization, e.g. overexpression of homologous and heterologous genes and industrial recombinant

strains of *T. reesei* by gene deletion, gene disruption and gene replacement, will be discussed later in Volume 2, Chapters 13 and 16.

10.5.1 Cloning by complementation

In comparison to *S. cerevisiae,* the lack of shuttle vectors in filamentous fungi makes it more difficult to clone genes by complementation. Several reports on cloning fungal genes in *E. coli* and especially in *S. cerevisiae* (Ballance, 1986; Goldman *et al.,* 1992; Margolles-Clark *et al.,* 1996a,b; Penttilä *et al.,* 1987a; Saloheimo *et al.,* 1994; Schrank *et al.,* 1991; Ståhlbrand *et al.,* 1995) have been published. With many genes from filamentous fungi, complementation of yeast mutants and/or overexpression of cDNA clones do not lead to satisfying results. This is due to the fact that fungal genes may not be expressed in these species and/or functionality of the genes may not be given by heterologous expression. Futhermore, isolation of specific cDNA for some genes, e.g. regulatory genes, can be quite difficult because of its low representation in the total mRNA pool. An alternate strategy to overcome these problems is cloning by gene complementation in the species itself. The major prerequisites for this attempt are obtaining or creating the appropriate mutants, a genomic library of a particular fungus, a high-frequency transformation system and a rescue system for the transforming DNA.

All transformation systems described for *Trichoderma* spp. and *Gliocladium* spp. show a moderate frequency and therefore it may be possible to screen a gene bank by using realistic quantities of DNA and protoplasts or conidia (Ward, 1991).

For cloning an *A. niger* invertase gene, Bergès *et al.* (1993) applied a cosmid rescue technique after transforming the *T. reesei* QM 9414 *ura5*$^-$. Having the efficient *ura3* and *ura5* transformation system in hand, the authors transformed their pyrimidine-auxotrophic strain with an *A. niger* cosmid library and screened for direct expression of the *A. niger* invertase gene. After sib selection, DNA from putative positive clones was isolated and packaged in an *in vitro* system. Cosmids were recovered by infection of a suitable *E. coli* strain where they could be selected by ampicillin resistance.

A second marker rescue system applied to *T. harzianum* and *G. virens* was reported by Lorito *et al.* (1993). Plasmids were rescued from the genome of transformants by digestion with a single cutting *BamH*I restriction endonuclease and subsequent infection of *E. coli*. A comparison of re-extracted plasmid DNA from transformed bacterial colonies with the original transforming plasmid showed perfect alignment.

10.5.2 Use of reporter genes for promoter analysis

In the broad range of organisms from bacteria to higher eukaryotes, reporter genes have been extensively used to characterize regulatory DNA sequences. The two most prominent reporter genes, the *ß*-galactosidase *(lacZ)* and the *ß*-glucuronidase *(uidA),* are favoured because of their high sensitivity and easy quantification assays. Both systems have also been applied to filamentous fungi. In 1993, first reports on the use of reporter genes in *T. reesei* were published. Penttilä *et al.* (1993) fused the *cbh1* (cellobiohydrolase I) upstream regulatory sequences to the *lacZ* gene and

made deletions towards the 3' end of the promoter. A region 500 bp upstream was identified as responsible for glucose repression. The first use of the *ß*-glucuronidase reporter system in *T. reesei* was reported by Stangl *et al.* (1993); the authors could show that a 615 bp fragment of the *cbh2* promoter when fused to the *uidA* gene led to GUS activity on the conidial surface, hence suggesting the presence of conidiation specific signals within the *cbh2* 5' non-coding sequences.

Another strategy of applying a reporter gene in *T. reesei* was presented by Mach *et al.* (1996). The authors joined a 538 bp *xyn1* (endo-*ß*-1,4-xylanase I) and an 850 bp *xyn2* (endo-*ß*-1,4-xylanase II) fragment of the 5' upstream regulatory sequences to the *hph* gene, encoding *E. coli* hygromycin B phosphotransferase and then subjected transformants to growth on media containing different carbon sources and different hygromycin B concentrations. A comparison of the transcription of the two native xylanase genes to the expression of the corresponding reporter proved that the respective upstream sequences apparently contained all information necessary for induction and repression of biosynthesis of these two xylanases in *T. reesei*. Having this system in hand, the upstream regulatory sequences for repression by glucose were localized by random and specific 5' deletions (Mach *et al.,* 1996). Removal of areas from the *xyn1*- and the *xyn2-hph* fusions also led to the identification of short 5' upstream regulatory sequences responsible for the specific inducibility of both genes (Zeilinger *et al.,* 1996).

10.6 Concluding remarks

During the last few years the development of transformation systems has been described for several *Trichoderma* spp. and also one *Gliocladium* sp. The transformation could be achieved with auxotrophic or dominant selectable markers. All presently available gene transfer systems lead to genomic integration of the transforming DNA with a moderate transformation frequency. This powerful tool combined with a set of easily selectable markers has permitted great progress in biotechnological application and molecular genetic studies.

However, it shall be noted that events occurring during and after transformation of *Trichoderma* and *Gliocladium,* such as DNA integration, copy number and recombination, are still poorly understood. Therefore, observed results are often difficult to interpret and must be based on the analysis of a high number of randomly chosen transformants. A straightforward strategy to simplify this problem would be the development of a targeted integration system.

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11

Asexual genetics in *Trichoderma* and *Gliocladium:* Mechanisms and implications

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11.1 Introduction

Variation in Deuteromycetous and Ascomycetous fungi has been of great interest to researchers since at least the 1920s (Brierly, 1931; Hansen, 1938) and is an issue about which everyone working with these fungi soon becomes aware. For example, strains in culture may change in morphology, lose ability to sporulate normally, or suffer altered or lost pathogenicity. Such alterations are well documented and the literature frequently has been reviewed (Hastie, 1981; Kistler and Miao, 1992; Parmenter *et al.,* 1963). *Trichoderma* and *Gliocladium* spp. are no exception in this regard. While teleomorphic stages of these fungi are known (see Volume 1, Chapter 2), most strains have no known sexual stages. Therefore, the genetic events that occur in these fungi during vegetative growth and reproduction are of primary importance in considerations of phenotypic expression of characters. Further, the nature of the changes that occur and their interactions within the thallus probably have substantial impacts on appropriate taxonomy of these fungi, on their abilities to adapt to changing environmental conditions, and on their interactions with other organisms, including plants, other microbes, and other strains of *Trichoderma* and *Gliocladium*.

Over the past 30 years, a number of mechanisms of asexual variation have been shown to occur in fungi, including mutation, chromosome plasticity and rearrangement, and changes in the genetic composition induced by plasmids and transposons. Further, asexual recombination arising from natural anastomosis or laboratory manipulation may occur. In addition, cytoplasmic inheritance factors such as changes in the mitochondrial genome, invertrons and mitochondrial plasmids may affect the genetic composition of strains. All cells of strains of *Trichoderma* and *Gliocladium* that we have examined are polynucleate, with some cells containing very high numbers of nuclei, although some conidia are uninucleate (Section 11.5; Harman and Hayes, 1993; Toyama *et al.,* 1984). This polynucleate nature makes some degree of heterokaryosis almost inevitable in any strain that has not recently arisen from a single conidium. We suggest that the level of heterokaryosis, and the nature of the events giving rise to heterokaryosis, have substantial implications

for our understanding of phylogenetic relationships within these fungi and to their ecological fitness and adaptability in nature.

The purposes of this review are to examine (1) the mechanisms giving rise to diversity within strains, (2) the arrangement and location of genetic elements within the thalli of these two genera, and (3) the taxonomic and ecological implications of these variations. Of course, many of the mechanisms of variation have been elucidated in other fungi and have been well reviewed. This chapter will first consider the general mechanisms of variation that have been described in Ascomycetous and Deuteromycetous fungi and then indicate how these findings apply to *Trichoderma*. There have been few studies on the nature of asexual variation in *Gliocladium* spp., but it would seem reasonable to assume that similar mechanisms have evolved in these two genera. Finally, we will attempt to synthesize this knowledge into a comprehensive organismal concept for the asexual genetic events in these fungi.

11.2 Asexual variation in the nuclear genome in the absence of genetic recombination

11.2.1 Overview

A number of asexual mechanisms have been identified in fungi that may give rise to variation in the nuclear genome. These include chemically- or physically-induced mutation occurring either naturally or artificially, the activity of transposons, the occurrence of circular or linear plasmids, and chromosomal polymorphisms. Mutation is a well-understood and widely-known phenomenon and will not be addressed here, but we will consider each of the other mechanisms.

11.2.2 Transposons

Transposable elements (transposons) have been identified in prokaryotic and eukaryotic organisms and are a common cause of spontaneous genetic changes possibly leading to mutations (Daboussi and Langin, 1994). The contribution of transposons to mutation may lead to pathogenic specialization and race specificity in phytopathogenic fungi (Daboussi *et al.,* 1992; He *et al.,* 1996). The occurrence of transposons in diverse organisms, i.e., plants, fungi, insects, protists, amphibians, worms and humans, suggests that they may have been present in ancestors common to them all (Kempken and Kück, 1996).

Transposons have two defined characteristics. They can move from one position to another in the genome and they are present in multiple copies in the genome (Boeke, 1989).

According to their mode of propagation, transposons have been divided into two major classes. Class I elements transpose by reverse transcription of an RNA intermediate. These transposable elements contain sequences that code for reverse transcriptase domains (He *et al.,* 1996). Class I can be subdivided into retrotransposons having long terminal repeats (LTRs) and the LINE (Long Interspersed Repeat Elements)-like elements (Ll) of retroelements without terminal repeats (Kinsey, 1990). Transposable elements in class I typically have higher copy numbers in the genome (Farman *et al.,* 1996; Kempken and Kück, 1996).

Class II elements transpose through a DNA-DNA mechanism and can be split into two sub-groups: elements with short inverted terminal repeats (ITRs) and those

with ITRs of variable lengths. These elements demonstrate direct excision prior to their integration into target sequences (Daboussi and Langin, 1994). The best known examples are the Ac-Ds elements of maize, discovered about half a century ago by Barbara McClintock (1947). Class I transposons have been identified in the following fungi: *Cladosporium fulvum* (McHale *et al.,* 1992), *Fusarium oxysporum* (Daboussi and Langin, 1994; Julien *et al.,* 1992), *Magnaporthe grisea* (Dobinson *et al.,* 1993; Hamer *et al.*, 1989), *Neurospora crassa* (Kinsey and Helber, 1989), *Candida* spp. (Magee, 1993), *Schizosaccharomyces pombe* (Levin *et al.,* 1990), and *Saccharomyces cerevisiae* (Boeke, 1989). Class II transposons occur in *Aspergillus niger* (Glayzer *et al.,* 1995), *F. oxysporum* (Daboussi *et al.,* 1992; Daboussi and Langin, 1994; Langin *et al.,* 1995), *M. grisea* (Kachroo *et al.,* 1994), and *Tolypocladium inflatum* (Kempken and Kück, 1996).

The presence of transposons in phytopathogenic fungi may have an effect on pathogenicity. Hamer *et al.* (1989) identified *MGR,* which is a repeat sequence from *Magnaporthe grisea* in the genome of a rice-infecting strain; MGR was present in more than 50 copies in the genomes of strains that infect rice but existed in fewer than ten copies in strains that infected other monocot hosts. He *et al.* (1996) reported a distinct biotype of the causative agent of anthracnose, *Colletotrichum gloeosporioides,* that demonstrated race specificity towards specific cultivars of the legumes in the genus *Stylosanthes*. This biotype was found to contain the class I transposon, CgT1. At least six different transposable elements have been found in *F. oxysporum*. Comparison of the distribution of these elements in different *Fusarium* strains has indicated a possible relationship between population structure and epidemiology (Daboussi *et al.,* 1992; Daboussi and Langin, 1994).

Fusarium oxysporum is extremely variable in morphology and pathogenicity. It is an important plant pathogen that infects over 100 plant species. It exists in many specialized forms, grouped into formae speciales and races, based on their ability to cause diseases on particular host plants. The molecular basis for this variability is unknown; however, since this species has no identified sexual stage, mutation seems to be the main source of change. The activity of transposons may contribute to mutations that lead to pathogenic specialization and race specificity in phytopathogenic fungi (McHale *et al.,* 1992). Transposons may be able to alter gene activity and expression. Sequence changes that correspond to footprints of excised elements are frequently found as point mutations (Kachroo *et al.,* 1994). Thus, transposable elements could contribute in nature to genetic changes and cause the high level of variability observed with respect to pathogenicity and virulence reported for many plant pathogens.

The transference of transposons is unknown. Studies with *Fusarium* (Daboussi and Langin, 1994), *Magnaporthe* (Farman *et al.,* 1996), *Colletotrichum* (He *et al.,* 1996) and *Tolypocladium* (Kempken and Kück, 1996) suggest that horizontal transfer is possible. Kinsey (1990) demonstrated the transference of *Tad* between forced heterokaryons in *Neurospora:* forced heterokaryons between *Tad* containing and noncontaining strains rapidly acquired *Tad* in the nucleus. In normal heterokaryons of *Neurospora,* nuclei do not fuse. However, experiments clearly showed that *Tad* was transposed between nuclei but that nuclear fusions did not appear to occur.

Transposable elements may be important in the evolution of new pathogenic races of plant pathogenic fungi by insertional inactivation of genes or by producing sites for recombination. Transposons have yet to be identified in *Trichoderma* or *Gliocladium* spp. However, the frequent occurrence of such elements in other similar

fungi suggests that they are likely to occur in these fungi. If so, transposons could be strongly involved in the variability and diversity seen in these genera.

11.2.3 Plasmids

Plasmids are common in prokaryotic organisms where they can affect the fitness and phenotype of the host. Plasmids have also been identified in eukaryotic organisms, mainly in filamentous fungi and sometimes in plants (Arganoza *et al.,* 1994; Griffiths *et al.,* 1995), but no naturally occurring plasmids have been found in animals. Most plasmids are typically found in the mitochondria (Samac and Leong, 1989), but in some yeast species, plasmids have been found within nuclei (Futcher, 1988) or in the cytosol (Schaffrath and Meacock, 1996; Shepherd *et al.,* 1987; Tommasino, 1991). A linear, non-mitochondrial plasmid has been identified in *Alternaria alternata,* but its location outside the mitochondria has not been identified (Shepherd, 1992).

The mitochondrial plasmids of filamentous fungi can be linear (1.1–9.2 Kb) or circular (0.8–5.2 Kb) and can be found in high copy numbers (Samac and Leong, 1989). Since they are not generally lost from laboratory strains, they appear to be stable elements. Mitochondrial plasmids do not appear to be needed for normal growth of host cells since only a few strains of certain genera house them (Griffiths *et al.,* 1995). These plasmids may encode DNA or RNA polymerases, reverse transcriptases, senescence, or may have no coding functions at all (Arganoza *et al.,* 1994; Chan *et al.,* 1991).

Linear plasmids, sometimes called invertrons, typically have terminal inverted repeats and may have 5' covalently-bound proteins. Linear plasmids may encode putative DNA and RNA polymerases related to viral and bacteriophage polymerases, respectively (Kempken *et al.,* 1992). They have been found in *Agaricus, Ascolobus, Ceratocystis, Claviceps, Fusarium* (at least five isolates), *Gaeumannomyces, Morchella, Podospora, Pleurotus* and *Neurospora* (Arganoza *et al.,* 1994; Samac and Leong, 1989). Linear plasmids are not derived from the mitochondrial or nuclear genome (Schaffrath and Meacock, 1996).

Circular mitochondrial plasmids can be derived from the mitochondrial genome and some evidence suggests that these plasmids contain sequence elements of group I mitochondrial introns (Collins and Saville, 1990; Nargang *et al.,* 1984). In *Neurospora,* however, circular mitochondrial plasmids have been identified that do not have detectable homology to the mitochondrial genome of the host (Griffiths *et al.,* 1990). Circular mitochondrial plasmids have been found in *Cochliobolus, Neurospora* and *Podospora* (Arganoza *et al.,* 1994; Samac and Leong, 1989).

The mitochondrial plasmids of *N. crassa* may be linear or circular. They may encode for the DNA and RNA polymerases and may increase the rate of senescence (Court *et al.,* 1991; Griffiths, 1992). *N. crassa* mitochondrial plasmids have been isolated and categorized into six homology groups: the circular plasmids Varkud, LaBelle, Fiji and VS and the linear plasmids *kalilo* and *maranhar* (Arganoza *et al.,* 1994).

Griffiths *et al.* (1990) examined the transmission of *kalilo* and *maranhar* from one strain of *Neurospora* to another through cytoplasmic contact by heterokaryotic transmission. They found that senescence was horizontally transmitted by heterokaryosis within the same strain and between species. Since no heterokaryons can form

between *N. intermedia* and *N. crassa,* interstrain transfer may have been via a transient fusion. They proposed that a strong incompatibility reaction occurred after transient fusion, which prevented heterokaryotic propagation. However, during this transient fusion between the two different species, plasmid DNA was able to survive the presumed incompatibility reaction. Heterokaryons were identified where both plasmids coexisted in the same coenocytic mycelium. Single conidial isolates from the heterokaryon also contained both plasmids, demonstrating that both plasmids were co-inherited, were compatible with each other and could coexist with either or both of the nuclear genotypes. No additive lethal effects were observed when both plasmids were present. The occurrence of heterokaryotic transmission in both species demonstrated that this was a potential model for the spread of plasmids in natural populations (Debets *et al.,* 1994).

Linear plasmids have been isolated in yeast including *Debaryomyces, Pichia, Wingea* and *Kluyveromyces*. They share characteristics of other linear plasmids described earlier. The best described linear plasmids are cytoplasmic killer plasmids, *pGKL1* and *pGKL2,* and are found in the dairy yeast *K. lactis* (Schaffrath and Meacock, 1996). Strains that contain a killer plasmid can secrete an oligomeric protein that inhibits and kills other strains (Tommasino, 1991).

As mentioned earlier, plasmids in filamentous fungi occur naturally and may encode polymerases, transcriptases, or nothing at all. However, linear plasmid-like elements have been found in numerous strains of *Fusarium oxysporum* f. sp. *conglutinans,* with three pathogenic races identified that contain these elements. Races 1 and 5 utilize cabbage as a host, while race 2 had specificity for causing disease in radish (Kistler and Leong, 1986). Thus, plasmids may be a factor in determining the host specificity of a pathogen.

Plasmids have been in mitochondrial DNA of eight of twelve strains of *T. viride* examined (Myers, 1991). These data suggest that plasmids may be common in *Trichoderma* but, so far as we are aware, other studies are not available.

11.2.4 Chromosome polymorphisms

The numbers and sizes of fungal chromosomes have been difficult to determine microscopically due to their small size (Zolan, 1995). The development of pulsed-field gel electrophoresis has permitted rapid advances in determining the chromosomal arrangements within fungi. In many fungi including *Trichoderma* spp., karyotypes of different strains of the same species differ remarkably in numbers, sizes, and the location of genes in them. Such changes in nuclear gene arrangement no doubt contribute to variability in fungi.

Perhaps the most remarkable example of chromosome polymorphism is in the yeast *Candida albicans*. Rustchenko-Bulgac *et al.* (1990) collected spontaneously-occurring strains with different morphologies, all derived from a single strain. Fourteen of these variant colonies were subjected to orthogonal-field-alternation gel electrophoresis (OFAGE) and transverse-alternating field gel electrophoresis (TAFE). Eleven chromosomal-sized bands were obtained from the original strain, but none of the variants possessed the same karyotype. Several different types of alterations were seen, including strains that lost some bands but acquired either increased amounts of DNA in a corresponding homolog or else acquired a new

band. Other strains contained multiple occurrences of duplications without loss of the corresponding homolog. One strain contained about twice the level of DNA per uninuclear cell relative to the parent or any of the other strains. The authors suggested that this frequent occurrence of chromosomal rearrangement provides a means of genetic variation in this asexual organism.

Numerous filamentous fungi also possess dramatically divergent karyotypes, although none seem to change as rapidly as *C. albicans* in the study cited above. Generally, chromosomal polymorphism occurs slowly in filamentous fungi and strains have remained stable over several years (Zolan, 1995). In some studies, even individual strains of *Candida* spp. remained of constant karyotype over hundreds of generations (Lott *et al.,* 1993). Nonetheless, very substantial variations occur between strains of the same species.

For example, representatives of the entomopathogenic genera *Beauveria* (Viaud *et al.,* 1996), the closely-related *Tolypocladium* (Stimberg *et al.,* 1992), *Metarhizium* and *Paecilomyces* (Shimizu *et al.,* 1993) all have been karyotyped using contour-clamped homogenous electric field (CHEF) gel electrophoresis. Nine strains of *B. bassinia* were examined (Viaud *et al.,* 1996) and were found to contain five to eight chromosomes ranging in size from 1.2 to 7.7 Mb. Not only were chromosomes of different sizes in different strains, but similar genes were found on differently sized chromosomes. For example, the chromosome bearing the *ß*-tubulin gene was, in different strains, 7.7, 5.4, 5.2, 4.8 and 4.4 Mb. Seven related *Tolypocladium* and *Beauveria* strains had from five to eight chromosomes, and their sizes ranged substantially between the strains (Stimberg *et al.,* 1992). Six strains of *M. anisopliae* var *anisopliae* were karyotyped and their karyotypes differed less than with *Tolypocladium* and *Beauveria* spp. All strains contained seven chromosome-sized bands, and the largest chromosome in each case was 7.4 Mb. However, each of the smaller six bands differed in size between strains; for example, the sixth chromosome (the second smallest) differed in size in each strain and sizes ranged from 2.0 to 3.0 Mb (Shimizu *et al.,* 1992). *P. fumosoroseus* appeared to be the least heterogeneous of these fungi: three strains were examined and each gave six chromosome bands upon CHEF electrophoresis. Further, all six bands were similar in size. However, there was heterogeneity in the location of *ß*-tubulin genes and for genes encoding rRNA (Shimizu *et al.,* 1993).

These ranges of diversity in karyotype are representative of a wide range of fungi. For example, very substantial karyotype differences were demonstrated for *Fusarium oxysporum* and other *Fusarium* spp. (Migheli *et al.,* 1993), for *Septoria nodorum* (Cooley and Caten, 1991) and *S. tritici* (McDonald and Martinez, 1991), and for *Leptospheria maculans* (anamorph: *Phoma lingam*) (Morales *et al.,* 1993) and *Nectria haematococca* (Kistler and Miao, 1992; Miao *et al.,* 1991b). Less variation occurred in *Aspergillus nidulans* (McDonald and Martinez, 1991). However, in sexually fertile strains, variation between strains was restricted to small chromosomes (less than 2 Mb). One small chromosome was discovered to be a supernumerary or "B" chromosome not required for vegetative growth. In sexual crosses, it was not inherited in a Mendelian fashion but instead was sometimes absent or present in an altered form in some progeny. While it does not encode genes required for vegetative growth, it is essential for pathogenicity since it is required for detoxification of a plant resistance factor (Miao *et al.,* 1991a).

Kistler and Miao (1992) have proposed a hypothesis concerning the occurrence and maintenance of fungal polymorphisms. Filamentous fungi may undergo sexual

recombination (i.e., meiosis) infrequently, rarely, or never. Meiosis requires pairing of like chromosomes, and so strains with highly divergent chromosome profiles are unlikely to be able to undergo chromosome pairing and may therefore be sexually incompatible (Harman *et al.,* 1993). Zolan (1995) further points out that various studies have found a low number of chromosome-length polymorphisms in sexual fungal populations. However, the small amount of information on the nature of chromosome polymorphisms makes it impossible to predict the absolute effects of these variations on sexual populations or interstrain fertility. However, it is reasonable to assume that high levels of chromosome diversity will limit, or preclude absolutely, sexual recombination. Therefore, once chromosome polymorphisms reach a certain level, strains should be genetically isolated and are likely to evolve as independent populations. Consequently, it should be possible to estimate the frequency of genetic recombination within taxonomic groups by examining chromosomal polymorphisms. The greater the diversity, the greater the barriers to sexual recombination and the more genetically isolated individual strains are likely to be.

Several studies examining strains of *Trichoderma* spp. have demonstrated that these fungi contain substantial chromosomal polymorphisms (Tables 11.1 and 11.2). Fekete *et al.* (1996) examined single strains of *T. atroviride, T. hamatum, T. harzianum* and *T. viride*. All strains contained six chromosomes, although sizes of chromosomes differed; further, the size of the chromosome containing a conserved chitinase gene differed somewhat between strains.

Greater chromosome polymorphisms have been found in other studies on *Trichoderma*. Mäntylä *et al.* (1992) examined chromosome banding patterns in the wild-type *T. reesei* strain and a number of laboratory strains prepared from it by

Table 11.1 Numbers and sizes of chromosome-sized DNA detected in *Trichoderma reesei* strains in different studies

Size (Mb)	*T. reesei* QM 6a[a]	*T. reesei* QM 9414[a]	*T. reesei* Rut C-30[a]	*T. reesei* VTT-D-79125[a]	*T. reesei* QM 9414[b]
7.4					—
7.0				—	
6.9	—	—			—
6.6					
6.3	—	—	—		
5.7			—		
5.5					—
5.2		—	—		
4.6					—
4.2	—	—	—		
3.7	—	—	—	—	—
3.4	—	—	—	—	—
3.2					—
3.0		—		—	
2.8	—				

[a] Data are from Mäntylä *et al.* (1992) using CHEF.
[b] Data are from Herrera-Estrella *et al.* (1993) using CHEF.

Table 11.2 Numbers and sizes of chromosome-sized DNA detected in *Trichoderma harzianum* and *T. viride* strains in different studies

Size (Mb)	*T. harzianum* Group A[a]	*T. harzianum* Group B[a]	*T. harzianum* ATCC 32173[a]	*T. harzianum* G108[a]	*T. harzianum* GH2[a]	*T. harzianum* IMI206040[a]	*T. harzianum* IMI206040[b]	*T. harzianum* T-95[c]	*T. harzianum* T-12[c]	*T. harzianum* 1295-22[c]	*T. viride* T-9[b]
7.3		=	—	=	—	=	—				
7.2	—			—							—
7.1		=									
7.0							—				—
6.9			=								
6.5	=	—	—		—	=	=				
6.0											—
5.7		=		—							
5.6	—					—	—				
5.4								—	—	—	
5.3											—
5.1			=								
4.7		=		=				—			
4.5										—	
4.2									—		—
4.0					—			—			
3.7	=					—	—				
3.5					=						
3.3					=						
2.7					—						
2.2						—	—	—			

[a] Data are from Gomez *et al.* (1997) using CHEF; chromosome bands shown as double lines were proposed by the authors to consist of two chromosomes based on densiometric analysis of the gel following ethidium bromide staining. Group A consists of three strains with similar banding patterns and group B consists of two strains.
[b] Data are from Herrera-Estrella *et al.* (1993) using CHEF; chromosome bands shown as double lines were proposed by the authors to consist of two chromosomes based on densiometric analysis of the gel following ethidium bromide staining.
[c] Data are from Hayes *et al.* (1993) and Harman *et al.* (1993) using TAFE.

mutation. Chromosome banding patterns differed markedly between the strains, and both different numbers and sizes of chromosomes were found (Table 11.1). Moreover, analyses of genes located on the chromosomes indicated very substantial shifts; for example, rDNA was located on chromosomes of 2.8 and 4.2 Mb (original strain QM 6a), 3.0 (QM 9414), 5.7 (Rut C-30), and 3.0 (VTT-D-79125), while a marker (RC11) prepared from a cosmid library was located on chromosomes of 4.2, 6.3, 4.2 and 7.0 Mb in size, respectively, for these same same strains. Gilly and Sands (1991) also have karyotyped the genome of Rut C-30 and obtained five chromosome bands, which is the same number found by Mäntylä *et al.* (1992); however, Gilly and Sands estimated the sizes of these chromosomes to be larger than those obtained by Mäntylä *et al.* Herrera-Estrella *et al.* (1993) examined one of the *T. reesei* strains examined by Mäntylä *et al.* (1992) and the two studies agreed on the number of chromosomes in this strain (Table 11.1).

Herrera-Estrella *et al.* (1993) also karyotyped a strain of *T. harzianum* and a *T. viride* strain and found that these strains differed in chromosome numbers and sizes (Table 11.2). Similarly, Hayes *et al.* (1993) compared three biocontrol strains of *T. harzianum:* (1) an auxotrophic mutant of strain T-95 which had undergone two rounds of mutation, first to produce the benomyl resistant phenotype (Ahmad and Baker, 1987) and then to produce lysine auxotrophy (Stasz *et al.,* 1988a); (2) strain T12 his- which had undergone mutation to produce histidine auxotrophy (Stasz *et al.,* 1988a); and (3) strain 1295–22 which was a prototrophic protoplast fusion progeny between T95 lys- and T12 his-. These strains all possessed a chromosome band of about 5.7 Mb, but strain T95 lys- gave three other bands of 5.7, 4.2 and 2.2 Mb, T-12 his- had another band of 4.5 Mb, and 1295–22 had another band of 5.7 Mb (Table 11.2). Interestingly, when Southern analyses were done using genomic DNA of strain 1295–22, no hybridization was obtained to the smallest chromosome of T95, but genomic DNA of T95 hybridized with all bands of the three strains (Hayes *et al.,* 1993). These data indicate that this smallest chromosome of T-95 is not represented in strains T-12 his- or 1295–22. The small size of this chromosome and its unrelatedness to the chromosomes of T-12 his- or 1295–22 suggests that it could be a “B” chromosome as discussed earlier for *N. haematococca*.

Gomez *et al.* (1997) compared ten strains of *T. harzianum* that possessed ability to control plant pathogenic fungi and that were collected from various places around the world. These ten strains exhibited substantial chromosome polymorphism, with six to nine chromosomes of varying sizes detected in these strains. However, two strains formed a group with similar-sized chromosomes and three strains formed another group (Table 11.2). These also appeared to be related when RFLP patterns were considered but these patterns differed in other strains. In addition, they probed the chromosome profiles and found that individual genes were located on substantially different sized chromosomes. For example, a gene encoding endochitinase was found on chromosomes differing in sizes of 6.5 to 7.3 Mb, depending on the strain examined.

Taken together, these data indicate both that mutation causes substantial chromosome rearrangement in *Trichoderma* spp. and that there also is substantial variation in the chromosome arrangements of different *Trichoderma* strains. It would be extremely useful to conduct larger studies of chromosomal variation within wild and genetically-altered strains of *Trichoderma* spp.

In all of these studies, the chromosome numbers should be considered a minimum number, since more than one of these large sections of DNA may be sufficiently

similar in size to band at the same location. This difficulty was recognized by both Herrera-Estrella *et al.* (1993) and Gomez *et al.* (1997) and they assigned doublet chromosomes to particular bands based on density of ethidium bromide staining (Table 11.2). No doubt in Hayes *et al.* (1993) the numbers of chromosomes are underestimated because of this phenomenon. Further, estimations of sizes of chromosomes probably are comparable within a particular study but differ between studies. Size markers at this large size are few and determinations will not be particularly accurate. However, there can be no doubt that there is substantial chromosomal polymorphism in *Trichoderma* and other fungal genera.

Clearly, then, very substantial variations exist in chromosomal arrangements of different strains of a wide range of fungi, especially those that primarily reproduce asexually. The mechanisms whereby this variation is induced have been considered by several authors and reviewed by Kistler and Miao (1992) and Zolan (1995). Kistler and Benny (1992) demonstrated that transformation could result in chromosome polymorphisms. They produced a linear plasmid with a repeated teleomeric sequence on one end and used this to transform *N. haematococca* to be resistant to Hygromycin B. They obtained strains in which the transforming sequence was stably integrated, but many strains had altered chromosomal banding patterns. Some of them lost a chromosome-sized band, which was replaced by a smaller band, that the authors attributed to a partial deletion of a substantial portion (up to 2 Mb) of chromosomal DNA. Other strains were obtained in which a chromosomal-sized band was lost but was replaced by a larger one. Presumably, this occurred as a consequence of chromosome breakage due to insertion and subsequent attachment of the fragment to another chromosome. Further, they described one transformant that contains all of the chromosomes of the wild strain plus an additional supernumerary chromosome of 430 Kb. They presumed that this small band occurred following integration of the plasmid into an internal site of one chromosome, loss of DNA distal to the centromere from the site of integration, and mitotic disjunction so that both the wild-type and the supernumerary chromosome were maintained (Kistler and Miao, 1992), although perhaps not in the same nucleus. Further, McCluskey *et al.* (1994) have demonstrated that in *Ustilago hordei,* a strain with aberrant morphology was obtained following heat shock. This strain apparently suffered a 50 Kb deletion in a 940 Kb chromosome. Zolan (1995) attempted to explain the apparent paradox between chromosome stability within strains and the frequent observation of chromosome polymorphism between strains. She suggested that a low level of mitotic rearrangement does occur in wild strains. In addition, physiological stresses, such as starvation, may relax the normal tight mitotic control and lead to more rapid chromosomal changes than usually seen in cultures maintained in laboratories.

In conclusion, chromosomal polymorphisms are common in fungi, including *Trichoderma* spp. They may be induced by a range of conditions, including mutation, integrative transformation events, and environmental stresses such as heat shock or even starvation. In the absence of meiosis and sexual recombination, such changes can be perpetuated in asexually reproducing strains. Of course, asexual recombination can also induce chromosome polymorphisms, which will be addressed in section 11.3.2 of this chapter. When sufficient changes in chromosome structure have occurred, sexual recombination probably is no longer possible and genetically-isolated, independently-evolving populations of particular species probably occur (Geiser *et al.,* 1994).

11.3 Variation in the nuclear genome resulting from asexual genetic recombination

11.3.1 Classical parasexuality

For many years, fungi have been known to undergo somatic hybridization. This work originally was developed and analyzed with sexually compatible strains of *Aspergillus nidulans* (Pontecorvo, 1956), and so the processes that occur within these closely related strains might be expected to differ from strains that rarely, if ever, undergo meiosis.

The classical process of parasexuality has been described in detail (Hastie, 1981; Pontecorvo, 1956; Tindale and MacNeill, 1969). In summary the steps are as follows:

1. Two cells from dissimilar strains fuse (karyogamy) either through the natural process of anastomosis (Tindale and MacNeill, 1969) or else cell fusion is induced by protoplast fusion (Peberdy, 1979) or by other laboratory processes (Sivan *et al.,* 1990).
2. The resulting thallus is a heterokaryon containing nuclei of both parental strains.
3. Two dissimilar nuclei in the heterokaryon fuse to form a recombinant diploid (Hastie, 1981; Pontecorvo, 1956).
4. The resulting diploid heterozygous nuclei multiply side by side with the parental haploid nuclei in a new heterokaryotic condition (Pontecorvo, 1956).
5. Eventually, a homokaryotic diploid thallus may sort out and become established as a strain (Pontecorvo, 1956).
6. Mitotic crossing over may occur during multiplication of the diploid strain (Pontecorvo, 1956). Mitotic "crossing over" could result from a number of processes; Zolan (1995) has summarized these. Possibilities include exchange between tandem repeats on sister chromosomes or homologs, chromosome breakage and healing between sequences from different chromosomes, ectopic recombination between repeated sequences on nonhomologous chromosomes, and recombination in subteleomeric regions leading to variability at chromosome ends.
7. Loss of chromosomes to give rise to a recombinant haploid strain (Hastie, 1981; Pontecorvo, 1956).

Classical parasexuality, as defined above, clearly has been shown to operate in some fungi. Various genetic markers, such as auxotrophy or toxicant resistance, have been shown to segregate in all possible combinations in fungi such as *Verticillium* and *Aspergillus* (Pontecorvo, 1956). Hastie (1981) listed 22 different fungi in the genera *Ascochyta, Aspergillus, Cephalosporium, Cochliobolus, Emericellopsis, Fusarium, Humicola, Penicillium, Pyricularia* and *Verticillium* in which there is evidence of parasexuality; evidence also has been presented for parasexuality in the entomopathogenic fungi *Beauveria bassiana* (Paccola-Meirelles and Azevedo, 1991) and *Paecilomyces fumosoroseus* (Riba and Ravelojoana, 1984).

Types of evidence used to establish the existence of parasexuality differ. One common and relatively convincing type of evidence is to produce parental strains

with multiple but different auxotrophic markers and then to isolate progeny that contain all or most of the markers in various combinations (Hastie, 1981; Paccola-Meirelles and Azevedo, 1991; Pontecorvo, 1956). In some species, diploids can be recognized since spores that contain diploid nuclei are larger, or, especially in genera with uninucleate cells, the quantity of DNA can be shown to be twice that of haploid parental or recombinant progeny strains (Hastie, 1981; Pontecorvo, 1956). In *Verticillium,* putative diploid nuclei have been observed cytologically as larger nuclei (Puhalla and Mayfield, 1974).

When this process occurs in fungi, several predictable different genetic types should be present in progeny strains. These include heterokaryons of various types, homokaryotic diploid recombinants, and recombinant aneuploids and diploids. In all of these there should be substantial quantities of the genetic materials of both parental strains in each progeny strain.

There has been a substantial interest in induction of parasexual events in *Trichoderma* spp. to produce recombinant strains with enhanced abilities over the parental strains to conduct various commercially useful processes, such as improved cellulase production in *T. reesei* (Toyama *et al.,* 1984) or improved biocontrol ability in *T. harzianum* or other *Trichoderma* spp. (Migheli *et al.,* 1995; Pe'er and Chet, 1990; Stasz and Harman, 1990; Stasz *et al.,* 1988a). In most cases, improvements in cellulase production (Toyama *et al.,* 1984) or biocontrol ability were obtained (Harman *et al.,* 1989; Pe'er and Chet, 1990; Sivan and Harman, 1991), although not in every case (Migheli *et al.,* 1995). These improvements are beyond the scope of this chapter, but Volume 2, Chapter 11 describes in detail the uses of one protoplast fusion progeny that has been commercialized for biocontrol purposes.

Further, Horwitz *et al.* (1985) investigated the photoreceptor system for conidiation in *T. viride* using asexual techniques. They made various mutants for photoreception, spore color and auxotrophy, allowed the mutants to undergo anastomosis, and then assayed the progeny. Using this technique, they obtained different complementation patterns and estimated that about 17 genes are involved in the photoreceptor system.

11.3.2 Asexual recombination in Trichoderma *species*

In this laboratory, we were interested not only in producing superior strains for biocontrol by asexual hybridization but also in the mechanisms by which these events occurred.

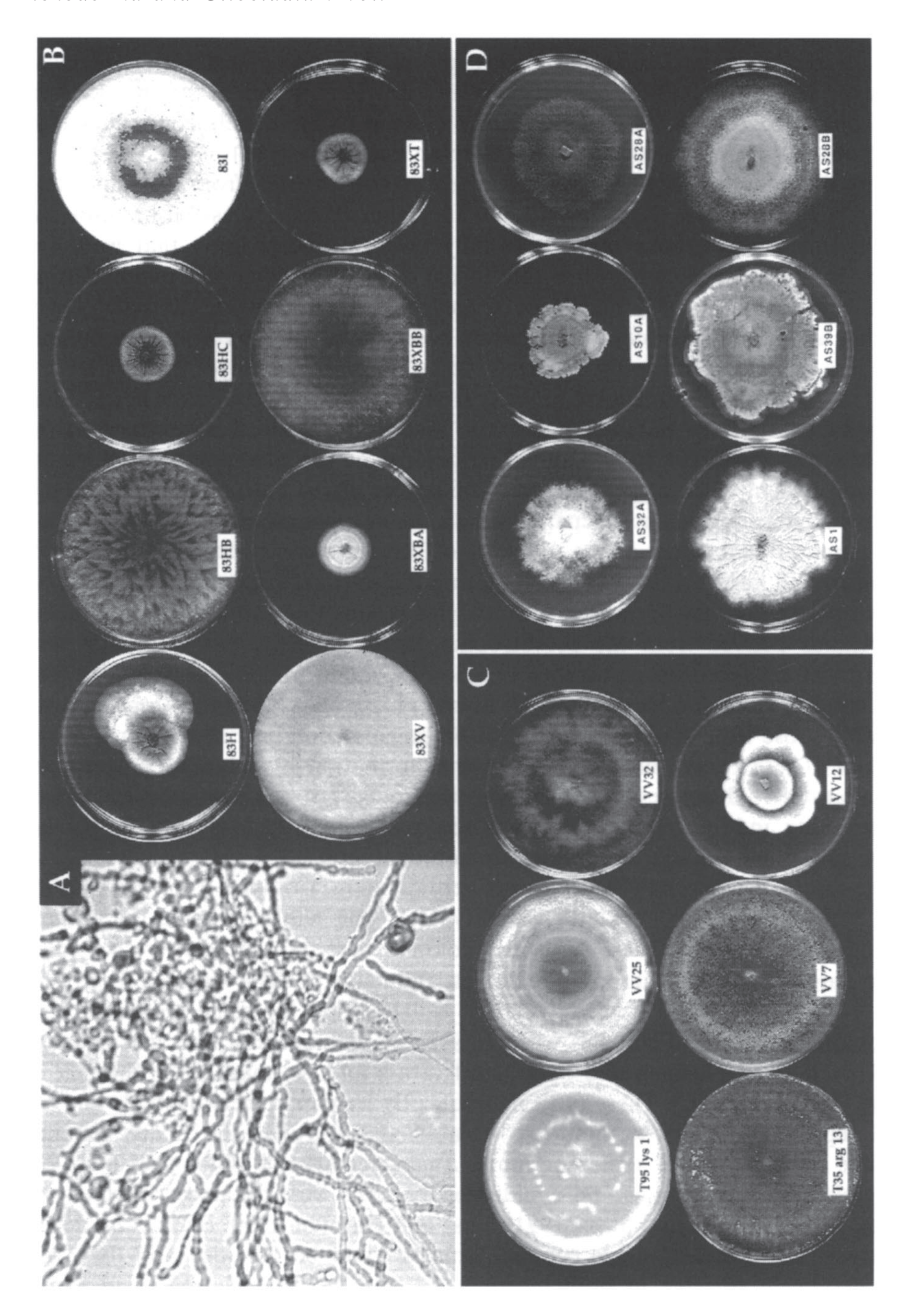
In most cases we induced heterokaryons using protoplast fusion, and complementation of auxotrophic mutants was used as the selectable marker. In order to accomplish this, we induced mutants with single or multiple nutrient requirements and selected for complementary strains that grew on minimal medium (Stasz *et al.,* 1988a, 1989). We also produced spore color mutants and selected for complementation as indicated by progeny that produced normal green conidia. Fusion between protoplasts was usually induced using $CaCl_2$ and polyethylene glycol and was monitored by fluorescent staining. In this latter procedure, protoplasts of each of the parental strains were stained using different fluorescent vital stains emitting light of contrasting colors; the two most useful stains for this purpose were hydroethidine

(red) and rhodamine 6G (yellow-green). The fusion methods were highly effective, and most fusants contained cells from both parental strains. In some cases only two cells fused, but frequently clumps of fused cells containing more numerous fused protoplasts were seen (Harman and Stasz, 1988).

Very different results were obtained when two auxotrophs from the same strain were fused relative to those obtained with fusion of protoplasts from unrelated strains. Intrastrain fusions resulted in fully prototrophic progeny that were very similar in appearance and growth rate to the original prototrophic parental strain (Stasz *et al.,* 1988a, 1989). Similarly, spore color mutants were allowed to grow together and spore color complementation occurred in the area where the colonies came into contact, presumably by anastomosis (Harman and Hayes, 1993).

All of these fully prototrophic progeny from intrastrain fusions were nearly indistinguishable from the parental phenotype and were balanced heterokaryons. No evidence was found for either diploid, anueploid or recombinant haploid strains. This was evident when suspensions of conidia were plated onto minimal medium or minimal media supplemented separately with the nutrient required for the growth of each parental auxotroph. Large numbers of conidia were evaluated (10^9 to 10^{10}) and nearly all gave rise to colonies requiring the nutrient required by the parental auxotroph. In very few instances, prototrophic strains were obtained from conidial suspensions derived from the prototrophic fusion progeny, but these also were found to be balanced heterokaryons in a second round of conidial isolations from these strains (Stasz and Harman, 1990; Stasz *et al.,* 1988a). The prototrophic strains probably arose from multinuclear hyphal fragments contained in the conidial suspensions. These results demonstrate (a) that formation of balanced prototrophic heterokaryons did not result in diploidization or any subsequent step in classical parasexuality and (b) that each conidium receives a single nucleus from the phialide and so that single conidial subisolates are homokaryotic.

The results of interstrain fusions were very different. Colonies from these fusions grew very slowly, only reaching visibility after one week or more on minimal medium (Figure 11.1A). These very small colonies were transferred to fresh medium to separate them from other fusant colonies, and many of them sectored to give rise to more rapid-growing colonies. Different isolates from the same groups of fused protoplasts gave rise to colonies with remarkably different morphologies (Harman and Hayes, 1993; Harman and Stasz, 1991; Stasz and Harman, 1990; Stasz *et al.,* 1988a) (Figure 11.1B,C). These progeny frequently were extremely unstable and sectored repeatedly. These sectors were isolated and regrown and many remained unstable for a period of a year or more. Again, remarkable differences in colony morphologies, growth rates and conidiation patterns were obtained (Harman and Hayes, 1993; Harman and Stasz, 1991; Stasz and Harman, 1990; Stasz *et al.,* 1988a) (Figure 11.1B). Further, single conidial isolates could be obtained and these also were extremely variable in both morphology and stability (Harman and Hayes, 1993; Harman and Stasz, 1991; Stasz and Harman, 1990; Stasz *et al.,* 1988a). In other experiments, we isolated nuclei from a prototrophic strain from one strain and fused these with prototrophs of a second strain (Sivan *et al.,* 1990). Only a few progeny were obtained, but these exhibited the same variability in morphology and stability exhibited by the progeny from protoplast fusion. Figure 11.1D shows variability between different single spore isolates from a single colony that was produced by nuclear transfer. Some strains eventually stabilize and can be used in commercial processes (see Volume 2, Chapter 11).


A
B
83I
83XT
83HC
83XBB
83HB
83XBA
83H
83XV
C
VV32
VV12
VV25
VV7
T95 lys 1
T35 arg 13
D
AS28A
AS28B
AS10A
AS39B
AS32A
AS1

To summarize, intrastrain protoplast fusion between two auxotrophic strains gives rise to fully prototrophic progeny similar or identical in phenotype to the original prototrophic parent. However, these progeny are quite different genetically from the parental strain since they are balanced heterokaryons and no genetic recombination occurred between these nuclei. Therefore, genetic events in *Trichoderma* are divergent from classical parasexuality. Further, interstrain protoplast fusion gives rise to great phenotypic diversity and results in very substantial strain instability. These effects are not a consequence solely of heterokaryosis since the same diversity occurs among single spore progeny.

Genetic analysis of the diverse progeny derived from protoplast fusion gave quite unexpected results. Extensive isozyme analyses were performed on the progeny using a range of enzymes that gave single, reliable and unequivocal bands of differing mobility for the parental lines and some of these chosen enzymes are dimeric in other organisms, which is an important consideration in these analyses. There is great diversity among isozymes of differing strains so these analyses are possible (Stasz *et al.,* 1988b). Over 1000 progeny were analyzed using multiple isozyme markers (Stasz and Harman, 1990; Stasz *et al.,* 1988a). Remarkably, in none of these was any evidence of genetic recombination observed. In a few cases, transient expression of two bands for particular isozymes were detected, but after further growth and sectoring, or single conidial isolations, these always resolved to single isozyme bands. Further, the multiple isozyme bands observed in any single progeny were always identical to that of one or the other parental strains. Frequently, sectors that arose from a single progeny were of different parental isozyme phenotypes, indicating that the initial thallus was heterokaryotic. In addition, even in the progeny that gave transient expression of a particular isozyme banding pattern from both parents, no heterodimeric banding patterns were ever obtained for dimeric enzymes. We interpret these results to indicate that this transient biparental banding pattern occurred as a consequence of heterokaryosis but that this heterokaryosis only rarely occurred within a single cell, so that heterodimeric enzymes were not formed in sufficient quantity to be detected in isozyme analysis (Stasz and Harman, 1990).

In contrast to the situation obtained with intrastrain fusions, the heterokaryons formed in interstrain fusions appeared to be very highly imbalanced. Usually, nuclei with the isozyme phenotype of one parent very largely outnumbered nuclei

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Figure 11.1 Development and variability in cultures of *Trichoderma* following protoplast fusion or nuclear fusion. (A) Appearance of typical interstrain fusion colony after about one week of growth on minimal medium. (B) Appearance of colonies derived from sectors of a single protoplast fusion progeny between *T. harzianum* T12 his^- and *T. harzianum* T95 lys^-ben^+. Note that strain 83A is sectoring; the variability shown in this figure was derived from similar sectors. All of the strains shown here are isozymically identical to strain T12 his^-, except for strains 831 and 83XV, which are identical to strain T95 lys^-ben^+. Published with permission from Harman and Stasz (1991). (C) Variation in progeny morphology between different progeny and the parents of the fusion between *T. harzianum* strain T95 lys^- and *T. harzianum* arg^-. Data on this fusion has been published (Stasz and Harman, 1990) but this figure has not heretofore been published. (D) Variation in single spore progeny derived from the fusion of nuclei from strain T12 with protoplasts of T95 lys^-ben^+. Data are from Sivan *et al.* (1990) but this photograph has not heretofore been published.

with the isozyme phenotype of the other parent. The genotype of the more numerous parental type will hereafter be designated as the *prevalent* genotype or parent and that of the less numerous type will be designated as the *nonprevalent* genotype or parent. In one fusion between *T. harzianum* strain T95 $lys^{-}ben^{+}$ (nonprevalent) and *T. harzianum* T12 his^{-} (prevalent), plating of single conidia on appropriate diagnostic media gave a ratio of 1:10000 or greater for the genotypes of two parental strains (Stasz *et al.,* 1988a).

Thus, these data indicate that classical parasexuality that includes recombination of large sections of the genome of the parental strains did not occur at detectable levels in these experiments. Instead, great instability and diversity is introduced in the absence of recombination. Further, this variation and instability is not a consequence of heterokaryosis since (a) it occurs in progeny with the isozyme phenotype of only one parent and (b) it is perpetuated and transmitted through single conidia.

These findings were extended using other genetic analyses. We analyzed chromosome banding patterns of two parental and one progeny strains from a fusion between the two *T. harzianum* strains noted earlier. These parental strains exhibit substantial chromosome polymorphism and so recombination of substantial portions of the genomes should have been detectable in the progeny strain (Hayes *et al.,* 1993) (Table 11.2). However, the progeny strain exhibited a chromosome banding pattern that was very similar to the prevalent parent T12 his^{-}, which also agreed with the isozyme phenotype of this strain. However, one chromosome band of the progeny strain appeared to be slightly larger than that of the T12 his^{-} parent (Hayes *et al.,* 1993) (Table 11.2).

We also examined both mitochondrial and genomic RFLP patterns of progeny and parental strains, as well as RAPD patterns from parental and protoplast fusion progeny (Hayes *et al.,* 1998b). Again, the results were in agreement with those of isozyme analyses. In only one case did the genomic RFLP pattern or the RAPD pattern differ from one or the other parental strain, and it appeared to be a heterokaryon. Further, the results of these analyses agreed with the isozyme phenotype already determined for the strain that was tested. We expected that the mitochondrial RFLP analyses would give a mixture of types since mitochondria and nuclei would be expected to be transferred independently in protoplast fusion, but this was not the case. In all progeny, the mitochondrial genotype matched that of the nuclear genotype, indicating that these organelles are not independently sorted as heterokaryons are resolved (Hayes *et al.,* 1998b).

11.3.3 Interstrain gene transfer, a new concept of asexual genetic recombination

These results pose a paradox. On the one hand, protoplast fusion induces great phenotypic diversity and instability. However, when genetic analyses are applied, the strains do not appear to have undergone recombination and, in fact, were extremely similar to one parental type or the other.

We have proposed a mechanism that we have designated interstrain gene transfer to explain these phenomena (Harman and Hayes, 1993; Hayes *et al.,* 1998a). We suggested that within interstrain but not intrastrain fusions many nuclei of the nonprevalent parent are degraded and that DNA fragments are produced. These fragments subsequently insert into the genome of the prevalent strain to give rise to novel genotypes. Further, it may be that these DNA fragments persist for a

time, perhaps as linear plasmids, and that the existence of these plasmids and transformation events may partially explain the instability of progeny strains. Further, the unusual phenotype of many progeny strains might be explained by insertion of sequences at sites that disrupt genes or regulatory sequences.

We determined to test this hypothesis and considered that we required a strain with a dominant selectable marker that could be identified using Southern analyses. We therefore transformed *T. harzianum* strain T95 his$^-$ with a gene for hygromycin B *(HygB)* resistance to give T95 his$^-$hyg$^+$. We determined that *HygB* was stably incorporated into the the T95 his$^-$ genome and that no detectable plasmids were propagated in the transformant. Protoplasts of this strain were fused with the prevalent parent T12. Progeny from this fusion were selected to detect prototrophic strains with the T12 parental phenotypic appearance but that expressed hygromycin B resistance. Such strains were readily detected and Southern analyses demonstrated that *HygB* was indeed incorporated into the genome of strain T12. RFLP and RAPD analyses were performed on this strain and all gave genotypes identical to strain T12. Thus, the concept of interstrain gene transfer, in the absence of classical parasexuality, was demonstrated to occur within *Trichoderma* spp.

To summarize, progeny derived from protoplast fusion exhibit great phenotypic diversity. However, the genetic events that occur in these progeny differ substantially from those of classical parasexuality as defined by Pontecorvo (1956) and others (Hastie, 1981; Tindale and MacNeill, 1969). Firstly, we obtained no evidence for karyogamy since diploid nuclei could not be detected. Secondly, we obtained no evidence for recombination of large genetic segments using isozyme electrophoresis, RAPD, or nuclear or mitochondrial markers. We postulated the existence of a process we have designated as interstrain gene transfer, in which the genome of one parental strain (the nonprevalent strain) is degraded to release small pieces of DNA. These DNA fragments then are integrated into the genome of the other (prevalent) parental strain. We proved the existence of this process by fusing an auxotrophic nonprevalent strain of *T. harzianum* containing *HygB* with a nonresistant prevalent strain. We selected for prototrophic hygromycin B-resistant strains similar in phenotype to the prevalent parent. These strains were readily discovered and were demonstrated to contain *HygB* in strains otherwise indistinguishable from the prevalent strain.

Other fungi probably also undergo interstrain gene transfer. For example, in heterokaryons of *Neurospora,* diploids do not form following asexual fusions indicating that karyogamy does not take place, although genetic material may be exchanged (Collins and Saville, 1990; Griffiths *et al.,* 1995; Kinsey, 1990). Genetic exchange may occur via anastomosis even in the presence of incompatible *het* gene combinations in the parental lines. With incompatible parental strains, highly imbalanced heterokaryons are produced that give rise to highly variable unstable progeny (Griffiths *et al.,* 1995), which is similar to the events we have identified in *Trichoderma*.

Even stronger direct evidence of interstrain gene transfer-like genetic events, as opposed to classical parasexuality, was recently described for *Magnaporthe grisea* (Zeigler *et al.,* 1997). This fungus undergoes anastomosis when strains are paired and gives rise to novel genotypes, as determined by extensive DNA fingerprinting analyses. However, “novel haplotypes were always very similar to one of the original isolates, showing only a few losses or additions of bands”. In other words, only small pieces of DNA from one strain introgressed into the genome of the

other parental strain, which is exactly the situation we observe in *Trichoderma* as a consequence of interstrain gene transfer.

The mechanisms of interstrain gene transfer are certainly not fully known. However, there are a number of intriguing possibilities. We do know that small pieces of nuclear DNA from one strain become integrated into the nuclear genome of another. This could occur spontaneously and immediately upon release of DNA from the nonprevalent strain. Even fragments of DNA from the entire genome of one strain may be introduced into the genome of a second strain following biolistic transformation (Lorito *et al.,* 1993). Thus, the mechanisms for ready and relatively immediate integration exist.

However, it is also possible that segments of DNA released from one strain may be modified and may replicate for various lengths of time within the recipient strain. In *Fusarium oxysporum,* a plasmid without ability to replicate was introduced into the fungus via protoplast-mediated transformation (Powell and Kistler, 1990). The plasmid was rearranged within the fungal thallus to produce self-replicating linear plasmids. These plasmids contained both rearranged plasmid DNA and other DNA acquired from the genome of the fungus. The plasmid DNA was larger than the original plasmid. The DNA from the parental strain included regions permitting autonomous replication and consensus teleomeric sequences. There were about 10 to 50 linear plasmids per nucleus in the strains tested (Powell and Kistler, 1990). If similar plasmids are formed in protoplast fusion progeny following heterokaryon formation in fungi undergoing interstrain gene transfer, different gene segments might survive as linear plasmids for substantial lengths of time. They could integrate into the genome at different times, thereby creating a strain with a mosaic of nuclei of novel genotypes produced via interstrain gene transfer and with extrachromosomal genetic elements in the form of replicating linear plasmids.

Other extrachromosomal genetic elements may also be transferred following or during heterokaryosis. Earlier we indicated that diploidization did not occur in *Neurospora* but that genetic exchange occurred nevertheless. One mechanism is via transposons. When strains with and without the *Tad* transposon were allowed to undergo anastomosis, heterokaryons were formed. Even though nuclei did not fuse, the transposons were in some manner transmitted to nuclei lacking *Tad* elements. It is possible that *Tad* transcripts are exported into the cytoplasm, as is normal for polyadenylated transcripts, and that by some mechanism they re-enter either of the two types of nuclei present in the heterokaryon. An alternate possibility is that full length cDNAs are synthesized in the cytoplasm for the polyadenylated transcript. If cDNA is synthesized in the cytoplasm, it would presumably be the intermediate that re-enters the nucleus for integration into chromosomal DNA. When heterokaryons were serially transferred, the load of acquired *Tad* elements appeared to increase, indicating that transposition was continuing in these heterokaryons, even after all of the native nuclei had acquired *Tad*.

Similarly, even in vegetatively incompatible strains of *Neurospora,* as defined by the *het* gene complex, both plasmids and mitochondrial chromosomes are transferred from strain to strain following anastomosis (Collins and Saville, 1990; Griffiths *et al.,* 1995).

Further, there is evidence that asexual gene transfer is not restricted to even the same species or genus. In our work, we found that interstrain gene transfer occurred as readily between species as within species (Stasz and Harman, 1990). In addition, *Parasitella parasitica* is a facultative parasite of the Mucoraceous fungus *Absidia*

glauca, i.e., it is a mycoparasite. *P. parasitica* is unusual in that it forms a cytoplasmic bridge between the two fungi. DNA from the parasite was transferred to the host during this exchange as evidenced both by complementation of auxotrophy in the host and by transfer of a plasmid containing a gene encoding neomycin resistance. The neomycin reistance gene was propagated on an extrachromosomal element in the host, but this plasmid was rearranged in *A. glauca* following its transmission from *P. parasitica* (Kellner *et al.,* 1993).

The evidence for interstrain gene transfer in *Trichoderma* has been thus far presented for protoplast fusion progeny. Therefore, an important question is whether or not these events occur in natural populations?

The primary issue is whether or not interstrain hyphal anastomoses occur. Clearly, once heterokaryons are formed by protoplast fusion (Stasz and Harman, 1990), when isolated nuclei are introduced (Sivan *et al.,* 1990), or even when naked genomic DNA is inserted via biolistic transformation (Lorito *et al.,* 1993), heterologous DNA is introgressed into the genome of the recipient strain. This introgression is the primary feature of interstrain gene transfer. Clearly, interstrain hyphal anastomoses do occur. This has been demonstrated by conidial color complementation in mutants of the same strain by several authors (Bojnanska *et al.,* 1980; Harman and Hayes, 1993; Horwitz *et al.,* 1985). However, this aspect of asexual genetics in wild populations of *Trichoderma* has been nearly totally overlooked insofar as we are aware. However, a recent paper (Gomez *et al.,* 1997) does address this point. They examined ten strains of *T. harzianum* from around the world relative to chromosome polymorphisms, RAPD analyses and vegetative compatibility. Strains that appeared to be closely related by either chromosome polymorphism or RFLP analysis were vegetatively compatible as evidenced by the formation of anastomoses in paired cultures regardless of their geographical source. However, in incompatible interactions, cell death and collapse occurred at the points of contact and anastomoses were not observed. Whether more rare events permitting occasional exchange of genetic material occurred in these incompatible pairings could not be ascertained from these data. However, as mentioned earlier, incompatible strains of *Neurospora* still were capable of exchanging genetic material (Collins and Saville, 1990; Griffiths *et al.,* 1995). Clearly, the nature and extent of exchange of genetic information in wild *Trichoderma* populations, and the extent of horizontal gene flow, is an area requiring further investigation.

11.4 Summary of asexual genetic variation in fungi

The information in the preceding sections demonstrates that there are a number of mechanisms for asexual genetic variation in fungi. Within single strains, mechanisms such as mutation and the activity of transposons create variability. Further, chromosomes of *Trichoderma* are highly polymorphic, and polymorphism in other fungi may be introduced by a variety of factors including mutation, transposons, and even starvation or stress. Extrachromosomal genetic factors such as linear plasmids, alterations of the mitochondrial genome, and mitochondrial plasmids may also create diversity. Finally, DNA from one strain may be introduced into the genome of another in the process of interstrain gene transfer. Importantly, this exchange apparently occurs in the absence of karyogamy. Once alterations in the nuclear genome

occur, then the altered nucleus is different in some fashion from its neighbors, and in polynucleated cells, a heterokaryon is automatically formed. As these changes continue over time, each strain should become a complex mixture of nuclei with differing genetic potential. In the absence of meiosis, such diversity can proliferate within strains. This genetic diversity within a single thallus would be expected to be influenced by the numbers and arrangement of nuclei within strains.

11.5 Nuclear arrangements within *Trichoderma harzianum* thalli

Surprisingly, we know of no reports of studies of *Trichoderma* on arrangements of nuclei within thalli. We have reported that nearly all cells are polynucleate (Harman *et al.,* 1993); this section will report specific information on nuclei within the thallus of *T. harzianum* strain 1295–22, which is a commercially available biocontrol agent (see Volume 2, Chapter 11) as visualized by Giemsa staining essentially by the procedure of Shirane *et al.* (1989) although staining times were varied for different preparations to obtain good differentiation of nuclei and hyphae (Figure 11.2).

Different portions of the thalli differ remarkably in both size and number of nuclei within each of them. Mature hyphae from colonies grown on potato dextrose agar were examined in the region where conidial formation was just beginning and also from the advancing edge of the colony. Hyphae were of two distinctly different sizes, with larger ones about 10 μm in diameter and smaller hyphae about 3 μm (Figure 11.2A,B). A few hyphae were intermediate between these two sizes, about 5 μm. Such hyphal polymorphism has been described in *Trichoderma* before; only the small hyphae are involved in mycoparasitism (Chet *et al.,* 1981). All of these hyphae were polynucleate: smaller hyphae contained 5–6 nuclei per cell while larger cells had very many nuclei, sometimes exceeding 50 per cell (Figure 11.2A,B, data not shown). Further, groups of nuclei in different locations had different appearances. Some were round, while others, mostly grouped together, were markedly elongate (Figure 11.2C). These elongate nuclei were all oriented to the long axis of the hyphae and arranged in V-shaped groups, reminiscent of flying geese. Such elongate nuclei have been described in other fungi and have been attributed to motile nuclei migrating to other locations due to action of cytoskeletal elements (Aist and Wilson, 1967), which probably also is the case in *T. harzianum* hyphae (Figure 11.2C). Phialide stalks had less densely-packed nuclei, with one or two between the junction of the supporting hypha and the lowest phialides. The flask-shaped cells from which conidiospores are formed contain a single nucleus (Figure 11.2D) that apparently divided to provide a single nucleus to the newly formed spores. The young conidia themselves were mostly uninucleate (Figure 11.2E), although more mature conidia may be multinucleate (Stasz *et al.,* 1988a). However, mitoses of nuclei within these cells could be seen, and surprisingly, following mitosis the conidia underwent cell division to give rise to two conidia (Figure 11.2E). Such cellular division has not, so far as we know, been described for conidia of *Trichoderma* or other fungi. Young hyphae formed from germinated conidia were also observed. These hyphae were all of the smaller diameter and cells were polynucleate, with two to three nuclei per cell (Figure 11.2F). However, the hyphal tips were larger than the rest of the hyphal strands and the tips appeared to be filled with multiple nuclei based on their size and the density of staining (Figure 11.2F).

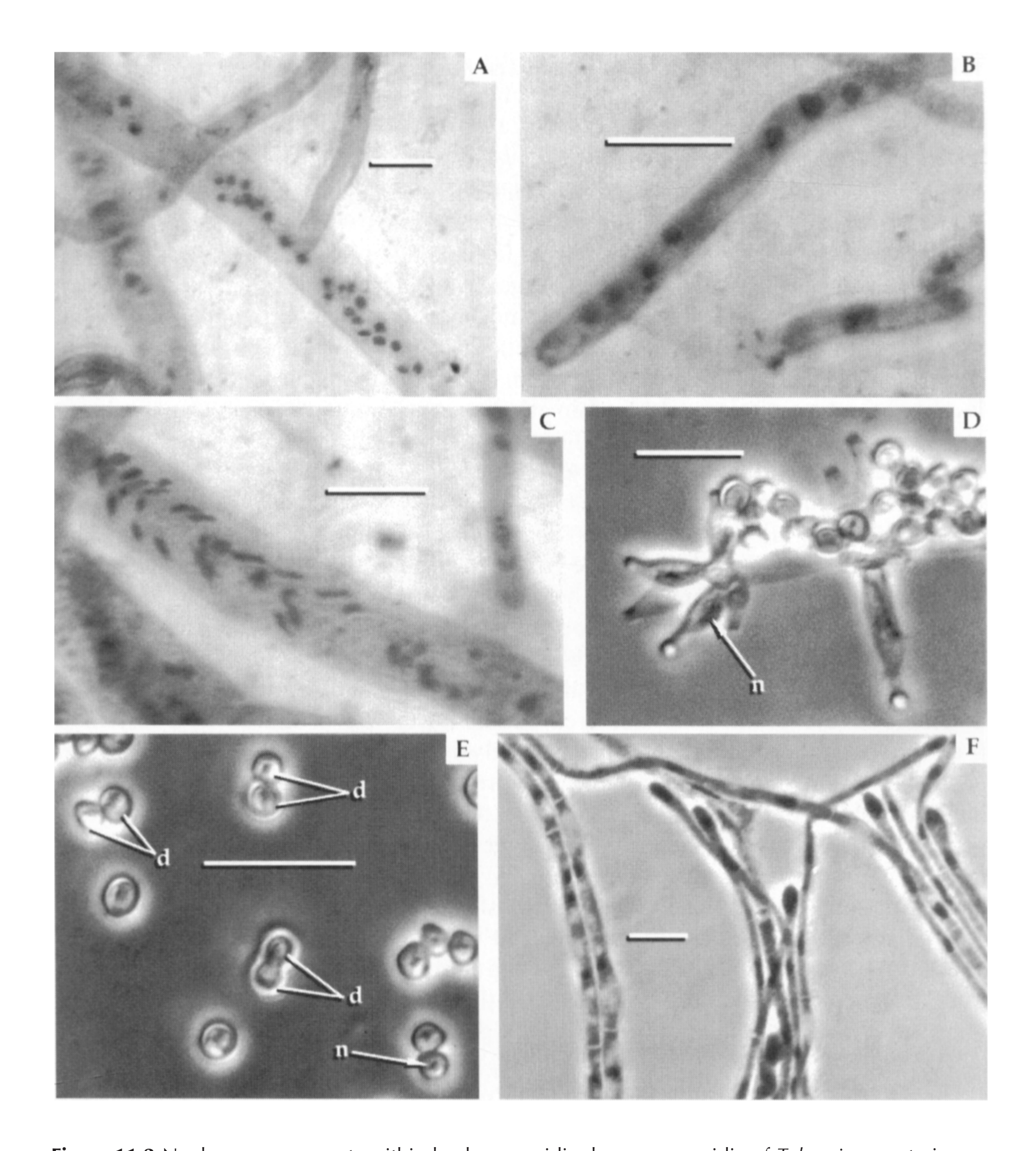


Figure 11.2 Nuclear arrangements within hyphae, conidiophores, or conidia of *T. harzianum* strain 1295–22. Nuclei were visualized by Giemsa staining essentially by the procedure of Shirane *et al.* (1989) although staining times were varied for different preparations to obtain good differentiation of nuclei and hyphae. In all figures, the bar represents 10 μm. (A, B and C) Hyphae from the advancing edge of a colony growing on potato dextrose agar. In (A) nuclei within a single wide hyphal cell (transversely arranged across the figure) are shown. In this and similar cells, more than 50 nuclei were present although the entire cell is not within the frame of this figure. In (B) one of the smaller hyphae from this region is shown; seven nuclei are clearly visible in this single hyphal cell. In (C) chromosomes in the large hyphal cell shown are arranged in a V-shaped pattern. (D) Nuclei in a phialide; there is a single nucleus (arrow labelled with "n"). (E) Conidia from the colony region where spores are changing from white to green. Division of conidia to form two cells is clearly evident (d), as are conidia each with a single nucleus (n). (F) Hyphae in germlings from conidia that germinated in potato dextrose broth. Multiple nuclei are evident in every cell, as are regions at the swollen hyphal tips that appear to contain multiple nuclei, based on the density of staining.

11.6 Implications of asexual genetics in *Trichoderma* relative to ecological fitness and taxonomy

These unusual genetic arrangements in *Trichoderma* and, probably, in other similar fungi, should have substantial implications for ecological adaptation and fitness. This chapter documents a number of mechanisms whereby the genomes of *Trichoderma* can be altered, frequently in fairly subtle ways. For example, chromosome rearrangements, absent from other genomic alterations, would leave the same genes in place but rearrange their chromosomal locations. Similarly, transposon movement and insertion would cause genetic changes localized in a specific area and leave the remainder of the genome unchanged. Interstrain gene transfer also causes localized changes within nuclei, but, in addition, nuclei of the opposite parental type may proliferate in a heterokaryon. Thus, both subtly-changed nuclei and nuclei of quite differing genotypes may be present in the same heterokaryotic thallus.

Equally importantly, such changes will occur only to one nucleus in a population of nuclei within individual cells and thalli. Since the nuclei likely can be motile, nuclei altered in one location may be transferred to another location. Perhaps more importantly, different changes can occur to different nuclei and so, over time, a highly diverse heterokaryon should occur potentially with each nucleus being subtly or substantially different from its neighbors.

Once heterokaryons are formed, altered nuclei may proliferate (undergo mitosis) at different rates than their unaltered counterparts. We produced hygromycin B transformants and maintained them vegetatively under selective pressure (Sivan *et al.,* 1992). When single conidia, which receive only a single nucleus from the phialide, were plated on media with and without the toxicant, only about 2% of the total germinable conidia were resistant. This suggested that transformed nuclei replicated more frequently than nontransformed nuclei. Apparently the thallus could retain its resistant nature even with low levels of transformed nuclei; the polynucleate nature of the thallus no doubt aids in this ability. Further, when these imbalanced heterokaryons (i.e., the mixture of transformed and nontransformed nuclei) were removed from selection pressure, the colonies lost their resistance to hygromycin B and numbers of nuclei containing the resistance gene dropped to very low levels (Sivan *et al.,* 1992).

To summarize, thalli of wild or successively transferred strains of *Trichoderma* spp. are very likely to be complex heterokaryons in the sense that individual nuclei may be different, albeit sometimes subtly, from their neighbors. Many different specific genotypes may exist, each differing from the others in chromosomal arrangements or specific localized insertions or other alterations. Of course, if anastomoses have occurred, some nuclei may be primarily of one parental genotype and others may be of the opposite parental type. Many, or probably most, of these parental nuclei may be altered by the process of interstrain gene transfer or other processes associated with asexual genetic recombination. In our work with protoplast fusion, we rarely found strains identical to the parental phenotype in growth rate, nutritional requirements and appearance. Some nuclear genotypes may more readily undergo mitosis than others, giving greatly imbalanced nuclear numbers. Further, extranuclear genetic alterations may further complicate this genetic mixture.

If these considerations are indeed true, then the thallus of a fungus such as *Trichoderma* may be considered a complex community of nuclei, some differing subtly and others differing markedly from their neighbors.

Strains containing a mosaic of nuclei in highly polynucleate cells should have a great advantage in adapting to environmental changes and stresses. It is very likely that nuclei with chromosomes in one conformation and with localized changes or additions to the genome will confer different advantages to the strain under particular selection pressures. This was readily evident in our work with protoplast fusion of various auxotrophic strains; we nearly always obtained complementation for the specific nutritional requirements in progeny by growing the strains under appropriate selective conditions (Stasz and Harman, 1990; Stasz *et al.,* 1988a). Similar results have been obtained by others when selecting for toxicant resistance, prototrophy, or spore color complementation (Bojnanska *et al.,* 1980; Migheli *et al.,* 1995; Pe'er and Chet, 1990; Toyama *et al.,* 1984). These data strongly indicate that almost any gene can be complemented or, in the case of heterologous genes, transferred from one strain to another via interstrain gene transfer. In other words, almost any gene may be transferred between nuclei via interstrain gene transfer; once selection pressure is applied, phenotypes are selected from the bank of diverse nuclei within individual thalli. The other mechanisms of variation in *Trichoderma* nuclear and extrachromosmal DNA provide additional mechanisms of variation.

This heterozygosity within individual thalli provides a potent means for *Trichoderma* strains to overcome environmental adversity. This ability may be enhanced by the large number of nuclei in each vegetative cell. No doubt many of the alterations within the genome are without value to the organism, but there are sufficient numbers that any deficiency in the gene products of one nucleus can be provided by another nucleus in the same or adjacent cells. Then, when environmental changes occur, nuclei coding for products conferring advantages to the new situation will be selected for and propagated. Thus, *Trichoderma* spp. probably do not rely upon a single genome to cope with environmental diversity but rather upon a relatively broad range of different genomic capabilities. The combination of the various mechanisms of genetic exchange and variation and the large numbers of nuclei per cell provide diversity of genotypes, which should provide a mechanism for very wide adaptations to diverse and changing environments.

However, this situation changes when conidiation occurs. As indicated earlier, conidia receive a single nucleus from the phialide and so are homokaryotic. Therefore, conidiation of a heterokaryotic thallus gives rise to as many separate and unique progeny as there are nuclear types in the thallus. This provides another avenue for the production and selection of new pheno- and genotypes in addition to selection that has already occurred in the thallus. However, this new generation now is composed of individuals that each possess a single genotype that may differ in larger or smaller ways from other individuals in the same population.

This variation may also have very substantial implications for the taxonomy of *Trichoderma* and similar fungi. The existence of both mechanisms for nonrecombinant and recombinant variants outside the sexual cycle should allow rapid changes in phenotype and produce genetically isolated individuals. The potential for rapid change in phenotype and genotype is obvious from the discussion in this chapter and the probable occurrence of heterokaryons further complicates this issue. The potential for genetic isolation as a consequence of chromosome rearrangement and subsequent difficulties in chromosome pairings in meiosis in fungi have been frequently discussed (earlier in this chapter; Geiser *et al.,* 1994; Kistler and Miao, 1992; Zolan, 1995). Geiser *et al.* (1994) considered similarity in chromosomal arrangements and other genotypic characters to be evidence of fairly recent sexual

genetic recombination. Conversely, substantial chromosomal polymorphism has been considered to be evidence that the strains are undergoing only asexual recombination and that this factor alone will necessitate exclusively asexual reproduction. If so, then asexual populations may evolve and become substantially at variance with populations that still are undergoing frequent or infrequent sexual recombination. Therefore, taxonomic schemes rooted in teleomorphic forms may not be appropriate for populations that have been genetically isolated from these sexually-reproducing forms. Most of this chapter has dealt with the diversity between and within strains; however, there is at least one report of remarkable homogeneity. Gomez *et al.* (1997) described substantial diversity within wild strains of *T. harzianum,* but did find two groups of strains that appeared closely related as measured by chromosome polymorphisms, RAPDs and vegetative compatibility. One group was composed of two strains from Israel, so these strains from a similar location might be expected to be similar. However, the other groups were composed of strains from different continents. Therefore, the strains in this group must have had some method of retaining a high degree of genetic similarity even after a substantial period of genetic isolation. One possibility to retain some genetic similarity is that the development of variability in these strains is restrained by some mechanism. Perhaps occasional sexual reproduction within these strains occurs, and this may serve to retain greater similarity within these strains, as has been suggested for *Aspergillus nidulans* (Geiser *et al.,* 1994).

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